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THESIS

Marine Microfouling in Monterey Harbor:
Observations Using the
Scanning Electron Microscope

by

James Earl Taylor

March 1977

Thesis Advisor: E. C. HADERLIE

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for 48 hours. A well-established community, including, metazoa, and many unidentified forms was formed during the first 3 weeks of immersion. Observations of microfouling upon aluminum, brass, and copper substrates were obscured by the formation of a thick crust, possibly of corrosion products.

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Marine Microfouling in Monterey Harbor: Observations Using the Scanning Electron Microscope

by

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Submitted in partial fulfillment of the
requirements for the degree of

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ABSTRACT

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I. INTRODUCTION

A. THE PRIMARY FILM

Marine fouling may be pragmatically divided into macro- and microfouling. Macrofouling consists of the metazoa and multicellular plants which settle upon structures man places in the sea, often interfering with their functions. Microfouling is the name applied to the organic monolayer and microorganisms, together known as the primary film or primary slime film, which settle rapidly upon virtually all submerged surfaces. It is composed of non-living organic material, bacteria, diatoms, yeasts, fungi, and protozoa in various proportions, sequences, and specific compositions depending upon the location, year and season, depth, proximity to previously fouled surfaces (O'Neill and Wilcox, 1971), and various physico-chemical parameters. It should be noted that this succession applies to the formation of fouling upon an initially clean surface. Though varying geographically, climax communities of fouling orgasisms are eventually established and remain stable for long periods.

The primary film itself is of direct importance in that it produces some frictional resistance (Woods Hole, 1952), can promote metallic corrosion (Kalinenko, 1959), and may, in the future, vitally affect thermal transfer through thin-walled heat exchangers of low delta-T in ocean energy extractors. The microfouling layer may increase a surface's frictional resistance by as much as 5%. This increase is, however, usually apparent only upon the substrate's initial movement through the water. Subsequent peeling of the film usually reduces resistance to levels not significant. Electrochemical corrosion of metallic substrate

may be accelerated by the presence of some bacteria through their chemical changing of initially non-corrosive ambient substances into ionic species capable of transporting electrons.

Of greater maritime importance, however, are the indirect effects of the primary film upon macrofouling. The fouling of submerged surfaces throughout the world follows a general pattern of ecological succession in which the presence of the primary film appears to be prerequisite, in some complex and incompletely understood way, to the settlement and growth of metazoa and multicellular algae. The primary film may favor the attachment of macrofouling by enmeshing free-swimming larval forms, discoloring bright or glazed surfaces, protecting fouling organisms from the toxic components of anti-fouling paints, serving as a food source, increasing the pH of the film-substrate interface (thus favoring the calcium-secreting forms) and/or by influencing the potential of the substrate's surface (Zobell, 1939). In addition to such manifest activities and relationships as the foregoing, the primary film appears in some manner to alter subtly the microenvironment of the substrate surface such that it is more favorable to succeeding organisms. Various researchers cite different components as being responsible for the environmental conditioning which allows the attachment and growth of subsequent settlers, but definitive relationships remain to be determined.

The effect of the film upon antifouling paints is also worthy of note. Primary film-forming bacteria and their extracellular polymeric products bind and precipitate soluble copper salts (Corpe, 1974b). The slime upon a paint surface can acquire, thereby, a large quantity of

that toxic metal, concentrating it to levels as much as three orders of magnitude greater than those found in a saturated seawater solution (Woods Hole, 1952). The film may thus improve paint performance by maintaining a high toxin concentration near the substrate surface, or by increasing the leaching rate through bacterial decomposition of the paint. It may, however, decrease the effectiveness of the paint if it concentrates the toxin in a combined, less poisonous form, or if the high concentration near the substrate surface decreases the dissolution rate of the toxin from the paint.

B. FORMATION OF AND SUCCESSION WITHIN THE PRIMARY FILM

The general pattern of biological succession upon a submerged surface has been described as beginning with the sorption of non-living organic material, followed by bacteria, then diatoms, protozoa, and, finally, algae and metazoans such as hydroids, bryozoa, crustaceans, etc. Clear definition of these phases at a site in a given year may be followed by a succession completely lacking in delineation a year later (O'Neill and Wilcox, 1971).

1. Sorption of Organic Matter

Free organic materials occur in seawater in both dissolved and particulate form. Dissolved organic materials originate as end-products of bacterial decay, excretory products, dissolution from broken seaweeds, etc., and consist principally of sugars, amino acids, urea, and fatty acids. The particulate component consists of detritus and delicate, plate-like aggregates ranging from 5 μm to several millimeters in diameter. These aggregates

are formed by the adsorption of dissolved organic matter upon bubbles and other naturally-occurring surfaces in the sea (Riley, 1963).

Baier (1973) has determined that the earliest events in the biological adhesion are influenced by the chemistry, texture, and charge of the substrate surface. He correlated the adhesion of biological entities with the "critical surface tension" (wettability) of the substrate. It should be noted that even such a biologically inert material as glass has some surface reactivity due to surface silica bonds which are free to attract hydroxyl, methyl, and amino groups. Baier (1973) suggested that the first acquired film was a glycoprotein layer 100-200 Å in thickness. He attributed the ability of this proteinaceous film to serve as an interfacial anchor for subsequently arriving cells to the drastic conformation and reactivity modifications attendant upon the transformation of the three-dimensional free-floating molecules to the two-dimensional form they took in the film.

Bacteria, in general, grow more rapidly and display more chemical activity upon solid substrates than while freely drifting (Zobell and Anderson, 1936). In addition to concentrating nutrients by adsorption, solid substrates may favor bacterial enzymatic activity by retarding the diffusion of exoenzymes from the vicinity of the bacterial cells (Zobell, 1943). The concentrated nutrients may, thereby, be more efficiently hydrolyzed by the bacteria's extracellular digestion.

While neither color nor plane seem to influence the attachment of bacteria (Zobell, 1943), the adsorption of dissolved material

is sufficient to change the suitability of a surface for subsequent biological settlement (Loeb and Neihof, 1975). Baier (1973) stated "in all instances, a prerequisite to adhesion of any cellular material to substrates is the prior accumulation of a predominantly proteinaceous 'conditioning film.'" The changes to the surface's suitability may be effected by increased nutrient concentrations, as described above, and/or by alterations to the substrate's wettability, surface charge, chemical bonding characteristics, and, perhaps, more subtle factors as yet unrecognized. The sorption of the organic film is important in altering the wettability and adhesiveness of solid surfaces and provides for strong chemical bonding with the mucopolysaccharides exuded by the film-forming bacteria (Baier, 1973; Baier, Shafrin, and Zisman, 1968).

2. Sorption of Bacteria

Aquatic bacteria live primarily attached to solid substrata or in colonial masses rather than as free cells (Bott and Brock, 1970; Zobell, 1943) and are common upon submerged surfaces within 1 day of immersion. Most sessile forms exude mucilaginous holdfasts; a few have stalks. Sechler and Gunderson (1973) identified up to 52 species, all heterotrophic, in a slime sample but most were transients and didn't persist; within days only a few species remained dominant.

The succession pattern among bacteria is, first, the attachment of common chemoorganotrophs which readily use the organic nutrients adsorbed to the solid substrata. These are followed by the somewhat more nutritionally specialized stalked and filamentous forms, which later become dominant (Corpe, 1973). Rods are adsorbed within 1 hour

of immersion of glass in seawater (Marshall, Stout, and Mitchell, 1971a). Zobell and Allen (1935) found that coccobacilli (ovoid in shape) of less than 1 μm in size were most numerous; slender bacilli 1-2 μm in length were also common. Most were gram-negative and had well-defined capsules 2-3 times as large as the enclosed individual bacterium. The dominant species were Achromobacter marinoglutinosus, A. membraniformis, and Flavobacterium amocontactus. Corpe (1972b, 1973) found 50-90% of the initial population consisted of motile, monopolarly flagellated bacilli of the genus Pseudomonas; 10-49% comprised a mixed group of yellow- or orange-pigmented Flavobacterium spp., and non-pigmented, non-motile rods of the genus Achromobacter. Coccoidal and spiral forms are rare but may appear after 6-8 hr (Zobell and Allen, 1935). Succeeding periphytes are stalked forms which may appear after only 24 hr of immersion (Marshall et al., 1971a), and may be dependent upon some condition created by the primary periphytes. Hyphomicrobium, Caulobacter, and Saprospira are genera which form a major part of the attached bacterial flora, C. halobacterioides and S. grandis appearing in numbers after 3 days and later becoming dominant (Corpe, 1972b, 1973; Starr and Skerman, 1965). Gorbenko (1966) found the dominant periphytic bacterial genera in the Black Sea to be: Vibrio (7 species), Achromobacter (2), Bacterium (2), Micrococcus (1), Sarcina (1) and Bacillus (1).

There appear to be three methods of bacterial approach to solid surfaces: (a) drifting, (b) electrostatic attraction, and (c) motility. Some non-motile forms may be carried randomly by water motion into the vicinity of solid substrates and fortuitously placed in contact with the surface. Electrostatic attraction of bacteria to surfaces covered

by the organic monolayer, however, is unlikely in that both the bacteria and the film are negatively charged (Corpe, 1970; Marshall, 1973; Neihof and Loeb, 1972). An implication of the high and rapid concentration of bacteria upon surfaces having an adsorbed organic film is that motile bacteria show positive chemotaxis and can detect adsorbed nutrients at a distance and move along a concentration gradient towards them (Marshall, 1973; Young and Mitchell, 1973b). The positive chemotactic response can be reversed by the addition of sublethal concentrations of toxic chemicals. This negative chemotaxis has been observed in a wide range of toxins, including heavy metals and hydrocarbons (Young and Mitchell, 1973a). Chet, Asketh, and Mitchell (1975) found that the most effective repellent organic compounds were acrylamide, benzoic acid, and tannic acid, all at concentrations not toxic to the bacteria.

The attraction of marine bacteria to the surface does not guarantee that all will firmly adhere. Small rods have been seen to have some selective advantage over other bacterial groups in the permanent sorption to surfaces. This advantage may result from a superior ability to multiply in the low-nutrient environment of natural seawater, or it might be related to the production of extracellular polymeric fibrils (Friedman et al., 1969, in Marshall et al., 1971a).

Surfaces coming in contact with the air-sea interface hold up to three orders of magnitude more attached bacteria per unit area than surfaces kept from contact with that interface (Sieburth, 1965, in DiSalvo, 1973). Rapid, irreversible sorption of thousands of bacteria per square centimeter occurred within 1-2 min of passing a surface

through the neuston layer into water typically containing 10^3-10^5 colonies per milliliter. This suggests that certain bacteria are pre-adapted or pre-conditioned for rapid attachment to surfaces of opportunity (DiSalvo, 1973).

The sorption of bacteria includes two distinct phases. First, reversible sorption, wherein the bacteria are very near, but not actually upon, the substrate, occurs. They are apparently in an equilibrium condition between electrostatic repulsion and such attractive influences as Van Der Waals forces, their own locomotive exertions, etc. Cells at this stage may be removed by washing or rinsing. This stage is followed by irreversible sorption among bacteria which can produce an extracellular bridging polymer, thought to be proteinaceous. When the protein synthesis of a film-forming pseudomonad was blocked by the antibiotic chloramphenicol, the bacteria did not attach (Marshall, 1973). While engaged in a rotational motion at the water-substrate interface, Pseudomonas sp. is able to produce polymeric fibrils which may be concerned in the irreversible sorption of the bacteria to surfaces (Marshall, Stout, and Mitchell, 1971b). Structural properties of bacteria perhaps related to their ability to attach to substrates include capsules, surface slime, pili, and holdfasts (Corpe, 1970). Capsule and slime polysaccharides are synthesized by many bacteria and may cement cells to solid substrates. These may be simple monopolymers or complex heteropolymers containing several different sugars linked in a variety of ways. Further, as a result of the capsule, the cell's surface charge may be altered (Corpe, 1970). Fletcher and Floodgate (1973)

stained marine bacteria with Ruthenium Red and Alcian Blue and demonstrated a compact extracellular, acidic polysaccharide layer involved in the adhesion of the bacterium to the surface. Once settled, the bacteria produced a secondary, fibrous, acidic polysaccharide which eventually replaced the primary polysaccharide. These carbohydrates are polyanionic and alcohol-insoluble (Corpe, 1973). Pili are long, straight, thin (100Å), hairlike structures, some of which are adhesive (Corpe, 1970), arranged perpendicularly to the cell's surface. Marshall et al., (1971a), however, believed it unlikely that pili were of significance in those bacteria responsible for the primary colonization of surfaces. Hyphomicrobium sp. produces long hyphal filaments with swollen ends by which it attaches to the substrate (Starr and Skerman, 1965). The caulobacters also attach by stalks and holdfasts. The wall of the stalk is continuous with the bacterial cell wall and is not a secreted material. At the end of the stalk is a holdfast material which is secreted and by which the attachment of the cell, perhaps irreversible, is accomplished. The chemical nature of the holdfast material is not known but appears to be neither carbohydrate not protein (Poindexter, 1964).

The attachment of the periphytic bacteria is so tenacious to glass slides that water may be poured from the slide-containing bottle and be replaced without dislodgement (Zobell, 1936). Corpe (1974a) found that attached bacteria could be removed by a 5-min exposure to NaOH, solutions of anionic and non-anionic detergents, chelating and oxidizing agents, or to protein denaturants. Distilled water, dilute buffers,

salt solutions and cationic detergents did not cause detachment. Meadows (1965), however, found that marine bacteria remained attached only in high salinities. The seawater could be replaced by NaCl solutions of the same ionic strength as seawater with no effect. A solution of glycerol of the same ionic strength, however, caused or allowed the detachment of many marine bacteria.

Corpe, Matsuuchi, and Armbruster, (1976), cultured primary film-forming bacteria upon various organic particles and glass slides. No apparent relationship between the growth and the chemical nature of the substrate was apparent. The Woods Hole study (1952) indicated that the total weight of the bacterial film ranged from 0.2-0.3 mg/cm^2 in winter to 4 mg/cm^2 in summer (Figure 1). As a general rule, the dry weight of the film amounts to about 30% of its wet weight. Of the dry weight, 10-25% is organic matter, the remainder being sea salts and insoluble ash (Woods Hole, 1952). The bacterial populations double approximately each 4 hr, depending upon the ambient temperature and available nutrients. Morales and Arias (1965) believed that the development of fouling has often been erroneously studied as a function of temperature and salinity. They considered the organic production of the water and nutrient concentrations to be of the greatest importance in bacterial growth. The development of fouling and the effective maintenance of anti-fouling paints may therefore be strongly related to primary production.

Sticky films formed by bacteria collect various kinds of debris, creating physically and chemically complex surfaces (Corpe, 1972a). Film-forming bacteria and other adherent cells retain enzymatic ability

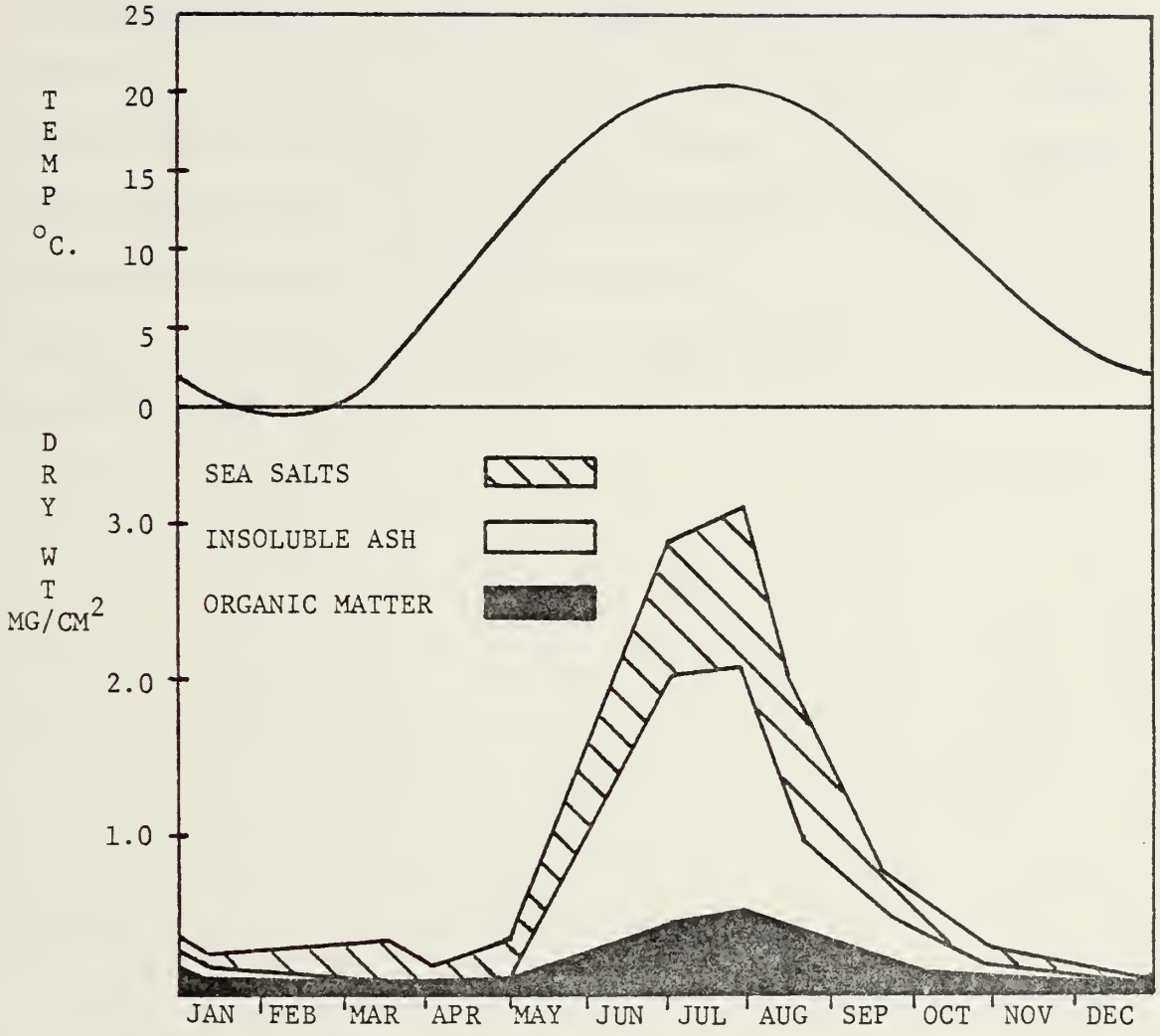

Figure 1. Weight and composition of slime films developed at Woods Hole, 1941. Above, the corresponding seawater temperature. (After Woods Hole, 1952)

long after their death so that microbial films upon solid objects may serve as centers of intense biochemical activity, perhaps making possible the development of communities of organisms which are unable to thrive upon clean surfaces (Corpe and Winters, 1972). It further seems very likely that the protein-polysaccharide film produced by bacteria would greatly improve the capacity of a glass surface, for example, to concentrate low molecular weight materials, such as carbohydrates, organic acids and amino acids (Duursma, 1965).

3. Diatoms

Diatoms, unicellular algae, become evident as early as six hours after immersion (O'Neill, 1971) but appear more generally after 4-5 days (Corpe, 1972a; Marshall et al., 1971a). O'Neill and Wilcox (1971) found diatoms to be the first microorganisms present in great numbers; bacteria were less significant.

Solitary diatoms, rather than colonial forms, are the earliest arrivals. Chain-like aggregrates of colonial diatoms follow soon thereafter and are eventually succeeded by stalked colonial forms, which become dominant (O'Neill, 1971).

Woods Hole (1952) listed the most common microfouling genera as Schizonema, Synedra, Licomorpha, Navicula, and Nitzschia. O'Neill (1971) found Cocconeis, Nitzschia, Navicula, Striatella, Surirella, and Pleurosigmia to be the common solitary genera; Grammatophora, Melosira, Acnanthes, Chaetoceros, were the early colonial forms; and Licomorpha was the dominant stalked genus.

In addition to the manifest environmental alterations brought about by such species as Cocconeis scutellum, whose slime and broken frustules form a crust later non-selectively colonized by a variety of microorganisms, diatoms also make subtle changes affecting the suitability of a substrate for subsequent attachment and growth of organisms (Sieburth and Thomas, 1973). In Madras Harbor, India, Daniel (1955) found that larvae of the barnacle Balanus amphitrite and the hydroid Hydroides norvegica settled upon glass slides only after the appearance of the primary film, which took 24 hr to form. In this case diatoms appeared to play a more important role than bacteria.

4. Other Microfoulers

In addition to the bacterial and diatomaceous components, the primary film contains protozoa, yeast, fungi, and small algae. The termination of the primary film phase of fouling begins with the settlement of metazoan larvae and the growth of multicellular algae.

a. Protozoa

The protozoan component reportedly arrives with, or shortly after, the arrival of significant numbers of diatoms, beginning at about 5 days of immersion (Marshall et al., 1971a). Off Pt. Loma, California, Corpe (1972a) found that test panels were covered with debris-encrusted Zoothamnium sp. after 7-9 days of immersion. The Woods Hole study (1952) concluded that the protozoan component was merely associated with the bacteria and diatoms but took no part in the film formation. These protozoa range in size from 0.002 mm to several centimeters. They may be motile or sessile; some are stalked. They multiply throughout the year but especially from May through October (Coe, 1932).

b. Algae

While later stages of multicellular algae are properly macrofouling, their spore and early multicellular stages, plus many unicellular forms, contribute significantly to the primary film. The order of importance of algal groups in fouling is red, green, brown, blue-green, (Woods Hole, 1952).

c. Metazoan Larvae

The larvae of such forms as bryozoans, tunicates, hydroids, barnacles, serpulids, and many more, live for a period in and, indeed,

appear to be dependent upon, the primary film. Metazoan encroachment upon the space occupied by the primary film organisms eventually reduces them to those periphytic forms which can live among or upon the dominant macrofoulers.

II. OBJECTIVES

Work on this research project was begun with the intention of developing techniques for the employment of the scanning electron microscope (SEM) in the observation of marine microfouling. Most prior research utilizing the SEM has consisted of the observation of particular species or closely related groups by individual authors. Furthermore, their work has been performed upon specimens cultivated in the laboratory or, in one instance, heavily polluted water. The SEM was to be used in this project by means of the techniques developed, to study the temporal succession within the microfouling communities upon the common shipbuilding materials such as aluminum, brass, copper, and stainless steel in the relatively unpolluted water of Monterey Bay, California.

Lebedeva and Shtevneva (1975) found that aluminum samples were less susceptible to bacterial fouling than were steel samples. Ships sunk in 1941 in the Black Sea's Egorlitskii Bay, Ukraine, were found to be heavily fouled; brasswork, however, was subject to light fouling (Sal's'kyyi, 1962). Four percent of the marine bacteria studied by Starr and Jones (1957) were stimulated by copper at a concentration of 0.2 mg/l. Copper may inhibit marine bacteria in a low-nutrient environment but where nutrient levels are high, its inhibitory effects are obliterated. Copper of a concentration of 4×10^{-4}M actually stimulates bacterial growth when nutrient levels are high (Corpe, 1975).

III. MATERIALS AND METHODS

A. EXPERIMENTAL SUBSTRATES

In order to use the SEM to observe the components of, and succession within, the primary films formed upon aluminum, brass, copper, and stainless steel, metal discs 1/2 inch (1.27cm) in diameter by 1/8 inch (0.32cm) in thickness (Figure 2) were machined to simulate the configuration of standard SEM specimen stubs. Each disc was drilled and tapped to receive a 1/8 inch (0.32cm) screw, which was inserted during microscopy to serve as a conducting pedestal. All discs were uniquely marked with an alphanumeric code by means of impression dies, then smoothed with successively finer grades of sandpaper, and given a final polishing with crocus cloth. The alloy compositions of the discs were:

metal	catalog #	composition
aluminum	63S (6063)	Si 0.2-0.6%, Fe 0.35, Cu 0.10, Mn 0.1, Mg 0.45-0.9, Cr 0.1, Zn 0.1, Ti 0.1, Other 0.1, and Al 96.6-97.45%
brass	none	Cu and Sn
copper	none	Cu with traces of Sn and Ba
stainless steel	303	Ni 8.00-10.00%, S or Se 0.15, Mo or Zn 0.60, Fe 67.10-71.10%

Structures for mooring the discs consisted of sections of polyvinyl chloride (PVC) piping, one meter long, which had been drilled to receive lead weights and polyethylene suspension lines (Figure 3). Nylon monofilament strings holding one each of aluminum, brass, copper, and stainless steel discs at 10 cm intervals were attached every 12 cm along the PVC piping.

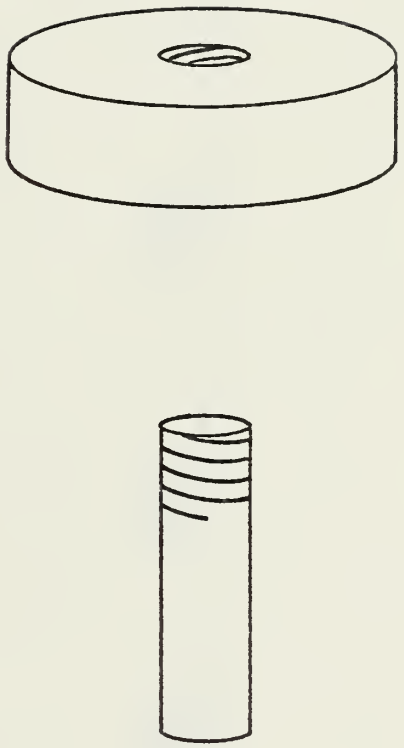

Figure 2. Specimen disc.

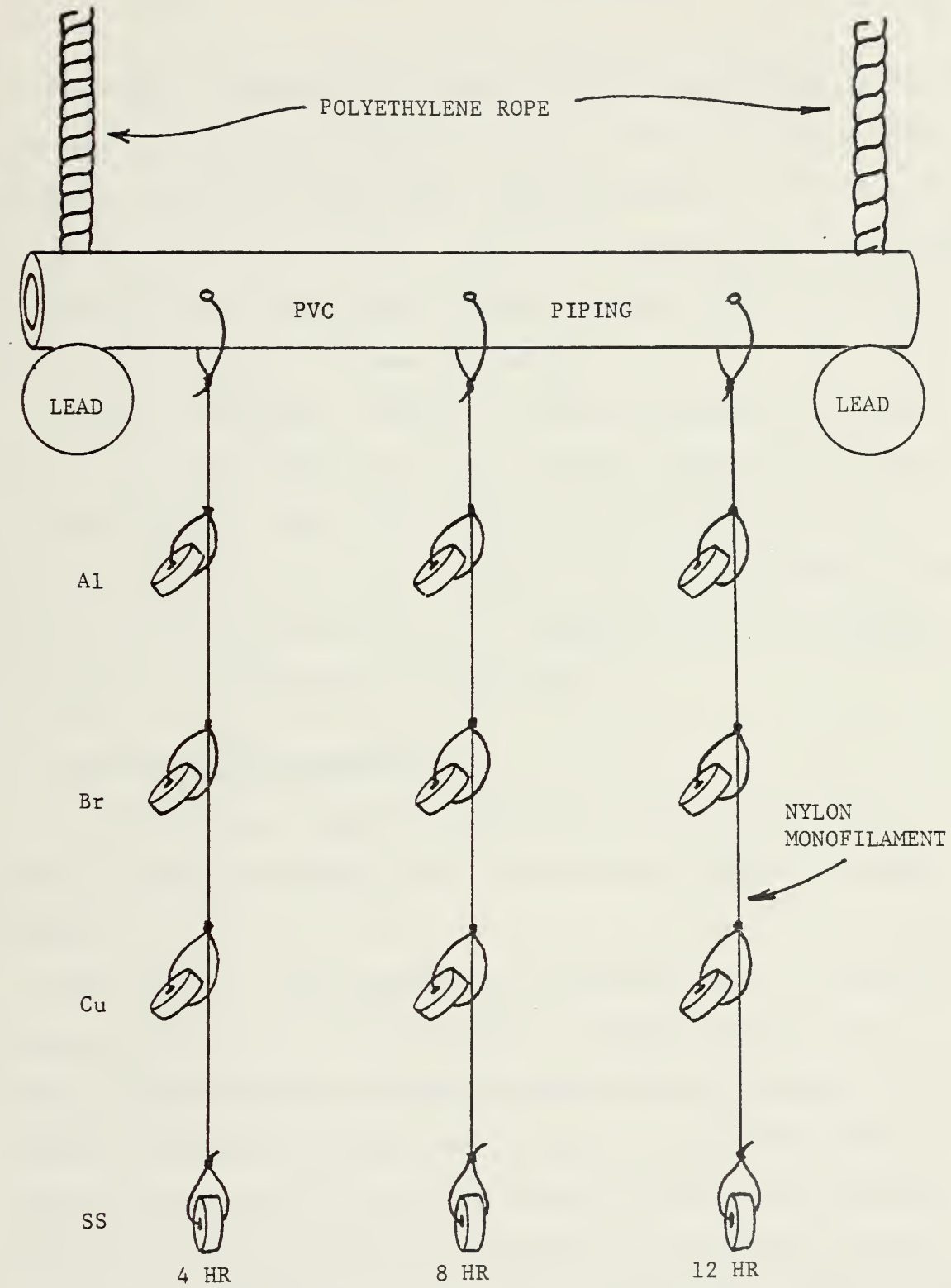

Figure 3. Mooring array.

The arrays of discs were suspended in Monterey Harbor beneath the tide station of Wharf #2 during the months of February and March, 1977. Strings of discs were removed after immersion periods of 4, 8, 12, 24, 48, 96 hr and 1, 2, and 3 weeks. During their immersion, the discs were in a shaded site at a mean depth of about 2 m below median lower low water in water of 13.5 ^{o}C. mean temperature.

To avoid artificially increasing bacterial populations, the disc arrays were passed through the air-sea interface sealed within a plastic bag, avoiding contact with the neuston at the air-sea interface. At appropriate times, individual strings of discs were plucked from the arrays and sealed within glass jars of seawater while still submerged, then taken to the laboratory for processing.

B. SCANNING ELECTRON MICROSCOPY

The SEM used was a model S4-10 Stereoscan, manufactured by Cambridge Scientific Instruments, Ltd., Chesterton Rd., Cambridge CB43AW, England.

Simply stated, a SEM consists of: an electron source, a focusing system, a scanner, an electron collector, a display system, and a means of transmitting the collected response from the specimen to the display. Primary electrons are accelerated in a narrow beam, from a cathode (source) through a series of electron "lenses" (Electromagnetic condensers) and are focused upon the surface of the specimen. Some of the primary electrons are backscattered while others enter the specimen, exciting its surface and causing the emission of secondary electrons. Changes in the surface composition, texture, or topography encountered

by the electron beam cause variations in the number, i.e., current, of backscattered and secondary electrons reaching the collector. These variations create, after amplification, a picture, in some sense an image of the specimen, upon the cathode ray tube (CRT) display. It can be arranged that the size of the raster[1] scan upon the specimen is very much smaller than the CRT of the presentation. Electronic expansion of that collected image to fill the CRT's field results in magnification, which may be as great as 100,000 times.

The advantages of scanning electron over optical microscopy are higher resolution (about 0.2 μm rather than 5 μm) and therefore higher magnifications, and great depth of focus, producing a "three dimensional" image. Its disadvantages are the inability to show internal details, the required vacuum environment (10^{-4}-10^{-5} torr) which precludes the observation of living specimens and necessitates the use of possibly artifact-inducing or shape-altering dessication procedures, and the lack of color response (Hearle, 1972).

The uniqueness of the SEM is its ability to produce a "three-dimensional" image. Two points must be borne in mind, however. First, this dimensional effect is an optical illusion which depends upon the interpretation of the observer for its effectiveness. Information about distance perpendicular to the plane of the image is not contained in the

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[1] A raster scan is one in which the scanning element is moved stepwise through a series of adjacent, parallel, straight lines, e.g., the eyes perform a raster scan across and down a printed page as the text is read.

picture itself; the observer infers the information by interpreting the image's lights and shadows. These deductions are fallible, and, for example, image areas appearing to represent concavities in the specimen surface often appear as convexities if the photograph is turned upside-down. Second, an image in a SEM is formed in a manner only analogous to that formed in a television; there are important differences. In an optical television system, the image focused upon the display screen differs little from that which would have been focused upon an observer's retina, i.e., an initial visual image is reproduced. The situation is different in a SEM; the brightness of each point of the image depends upon the number of electrons collected from the corresponding point on the specimen. The number of electrons collected is related to the efficiency of a given area of the specimen surface in backscattering electrons and not to its ability to reflect light. Thus, the brightnesses of two areas viewed by the SEM will probably be quite different than if those areas were to be observed visually by reflected light. The observer, however, tends to interpret the photograph as if it had been taken optically, i.e., as if he were viewing the specimen directly.

C. FREEZE-DRYING

A model 10-141 Unicool, manufactured by the Virtis Company, Gardiner, N. Y. 12525, was used as a final dessicator in specimen preparation. Freeze-drying involves rapidly freezing the specimen and removing the frozen liquid by sublimation under a high (10^{-5} torr) vacuum. This is done to minimize tissue damage or distortion by the surface tension forces which would occur as an evaporating liquid recedes.

D. GOLD PLATING

In a conductive specimen, e.g. a metal, most electrons from the SEM's incident beam are conducted through the specimen, into the stub, through the pedestal, and away to ground; only a relative few are back-scattered to the collector to form the image. Non-conductive specimens, however, such as organic materials, cannot rapidly channel the excess primary electrons away from the scanned area and a local charge is built up on the specimen's surface, increasing abnormally the secondary electrons emitted to the collector and creating a localized glow which destroys the imaging of the microscope. To avoid this condition, an extremely thin conductive coating may be applied to the specimen by vaporizing a conductor in an evacuated chamber holding the specimen. It was found that a 50 Å coating of gold reduced charging to an acceptable level without obscuring the surface with excess extraneous material. A 25 Å coating was applied in preliminary testing with less satisfactory results.

The plating process was accomplished in a Veeco 4E-401 Vacuum Evaporator, manufactured by Vacuum Electronic Corporation, Plainview, Long Island, N. Y.

E. X-RAY DIFFRACTION ANALYSIS

Analyses of the copper and brass alloy compositions were done by means of x-ray diffraction within the SEM using a PGT-1000 Analyzer manufactured by Princeton Gamma Tech, P. O. Box 641, Princeton, N. J. 08540. The PGT-1000 is attached to the SEM and measures the energy levels of the x-rays emitted by the specimen during bombardment by the electron

beam, compares those levels to a library stored within its computer, and displays the elemental analysis.

F. FIXATION

Primary fixation was accomplished using a 2% solution of glutaraldehyde (glutaric dialdehyde) in filtered seawater.

The aim of fixation is to preserve every detail of cellular ultrastructure exactly as it was in life. This ideal situation demands that all of the processes of life be suspended in an instant of time, that the semiliquid contents of the cell and its surrounding material be instantaneously solidified without disruption, that the relationships of every organelle and every molecule be preserved exactly as they were at the instant of fixation, and that any process likely to destroy the structure after fixation, such as autolysis (self digestion) or attack by microorganisms be prevented. The structures must also be preserved in such a way that the various processes involved in dehydration, etc., do not remove any components, add unwanted ones, or distort relationships.

Aldehydes penetrate fairly rapidly and preserve structure excellently by cross-linking free amino groups and toughening the tissues, minimizing the degree of collapse which usually occurs in the ensuing dessication procedures.

G. STAINING

In optical microscopy, certain dyes are frequently added to specimens to make them more easily visible or to distinguish between different

tissues to which the dye(s) may be selectively attracted. In scanning electron microscopy, analogous differentiation may be provided by differences in electron contrast, a function of physical density. Aldehydes, however, are very poor at providing electron contrast and the specimens were therefore treated with a 2% solution of osmium oxide (OsO_4) in filtered seawater. Osmium, the densest of all materials, is the best substance known for providing electron contrast and is also a fixative which is extremely effective in preserving the phospholipoprotein membrane skeleton of a cell. Although osmium oxide penetrates very slowly, it combines chemically with practically all cellular constituents and osmium metal remains behind in the fixed cell, attached firmly to the structures, stabilizing and delineating them almost perfectly.

Osmium oxide is very volatile and the vapor is intensely and unpleasantly toxic. As an excellent fixative, it kills any cells with which it comes in contact. It is especially damaging to the corneal epithelium of the eyes and the mucous epithelia of the nose and mouth. Contact can produce blindness and other effects, the full extent of which is incompletely known. It must always be handled with rubber gloves, in a fume hood with the glass front pulled down as far as possible, and the extractor fan turned on at full power. A wise added precaution is the use of goggles.

IV. SPECIMEN PREPARATION AND HANDLING

After removal from the sea, individual discs were removed from the strings in the laboratory and placed in 10 ml pyrex beakers containing filtered seawater. The beakers were then placed in an icewater bath and brought to a temperature of 0-4 °C., at which the specimens were maintained until freeze-dried. The seawater was gently drained from the beaker and replaced with pre-chilled 2% glutaraldehyde by means of micropipettes, as were all subsequent bath changes.

After 2 hr of fixation in glutaraldehyde, the steel discs were bathed for 3 hr in a filtered seawater rinse; the aluminum, brass, and copper discs were, instead, immediately triple-rinsed in distilled water, their beakers were drained and then plunged into liquid nitrogen (boiling point -196 °C.) for 4 min and, finally, placed into the freeze-dryer.

The rinsed steel discs were treated for 30 min in 2% osmium oxide and then subjected to a graded series of acetone-water dehydration steps, as follows: 10% acetone - 15 min, 30% acetone - 15 min, 50% acetone - 15 min, 70% acetone - 30 min, 90% acetone - 30 min, and 100% acetone for two 30 min periods. After draining the final 100% acetone bath, the beakers were plunged into liquid nitrogen and placed in the freeze-dryer.

After 4 hr or more of freeze drying, the aluminum, brass, copper, and stainless steel discs were plated with 50 Å of gold, completing the pre-microscopy preparation.

The prepared discs were examined in the SEM at an accelerating voltage of 20 kV and magnifications of 20-12500X. Photographs were taken by an attached camera, using Polaroid 55P/N film.

V. OBSERVATIONS

A. ALUMINUM, BRASS, AND COPPER

The original objective of observing and comparing the composition and succession of microfouling upon aluminum, brass, copper and stainless steel could not be realized. Natural chemical processes, probably corrosion, occurring upon the aluminum, brass, and copper discs so obscured the surfaces that effective observations could not be made. All copper discs, including that having been immersed for only four hours, were covered with a translucent film apparent to the naked eye. Brass discs immersed for more that 12 hr were visibly tarnished. No film coating was immediately apparent upon the aluminum discs but corrosion scabs were evident after 1-2 weeks.

Observation of the aluminum, brass, and copper discs by the SEM revealed a singular sparcity of organisms or even debris (Plates 1.a, 1.b, and 2.a.). It is postulated that those encrusted surfaces are the results of the presence of corrosion films which were transformed from flocculent or viscous gels into crusts during desiccation.

Initially, all discs were processed in exactly the same manner as that described above for the steel discs. After short periods, however, heavy deposits were visible on all non-ferrous discs and most especially upon those of aluminum. Processing was modified to drastically reduce the amount of time the non-ferrous metals remained in liquids. Although the new procedures significantly reduced the amount of corrosion product, there were still sufficient quantities to, in general, mask the disc surfaces from microscopy.

1. Aluminum

Bacteria were never detected upon aluminum. They may have been embedded within the crust as it formed from the collapse of the corrosion products during desiccation.

Solitary diatoms were rare. One was found upon the disc which had been immersed for 3 weeks (Plate 2.b.).

Chain-aggregate colonial diatoms (Plate 3.a.) were evident upon all discs immersed 4 days or longer.

A colony of unidentified, ovoid, stalked organisms (Plates 3.b. and 4.a.) formed upon the disc immersed for 3 weeks. The colony consisted of more than 50 individuals, each secured firmly to the substrate by a fibrous holdfast (Plate 4.b.).

2. Brass

The crust upon the brass grew more slowly than that upon the aluminum. After 3 weeks it had acquired a layered structure with a relatively smooth exterior and fibrous interior (Plate 5.a.). The only organism discovered upon the brass surfaces was a single bacterium (Plate 5.b.) on the disc which had been immersed for 48 hrs.

3. Copper

The copper discs remained remarkably smooth, although crusted over, and never gained identifiable life forms. The object in Plate 6.a. may have been of organic origin and represented one of the few objects to settle upon the copper surfaces.

B. STAINLESS STEEL

The stainless steel discs accumulated a sizable and diverse population by the end of the third week of immersion and the succession loosely followed that which had been anticipated. Of interest, however, is the fact that nearly all arrivals occurred later than expected. For reasons unknown, the development of the microfouling communities was very slow.

Although the steel discs began accumulating debris immediately (Plate 6.b.), it could be seen, in clear areas, that the steel surface remained void of any crust similar to those which formed upon the other test metals.

A single bacterium (Plate 7.a.) was detected upon the disc immersed for 4 hr but no others were discovered until examination of the disc immersed for 48 hr. Even patches of recognizably organic detritus (Plate 7.b.) were free of bacteria.

Appearing upon the 8-hr disc, and never seen again after the 24-hr disc, were aggregates of significant numbers of angular bodies (Plate 8.a.), possibly crystals. They occurred frequently in masses far larger than that shown in Plate 8.a. but always appeared within or upon grainy patches of unknown composition.

The first solitary diatom (Plate 8.b.) was discovered upon the 8-hr disc and was followed by moderate numbers of several forms (Plates 9.a. through 10.b.). Almost all were pennate; several skeletons of centric forms (Plate 18.b.) were also found.

The earliest colonial diatoms (Plate 11.a.), of chain-aggregate form, were detected upon the 24-hr disc. Subsequently, they

increased steadily in numbers, forming, by the third week, a substantial portion of the microbiota. Stalked colonial diatoms were never observed.

Bacteria were first detected in large numbers upon the 48 hr disc. The first significant arrivals were long, slender bacilli (Plates 11.b. and 12.a.). By the end of the second week, volcano-shaped bodies similar to those described by Gerchakov et al. (1977) as hemispherical bacteria had appeared. Cocci appeared in numbers upon the 3-week disc (Plate 12.b.).

Remarkably, protozoa were preceded by metazoa, but in small numbers. After 96 hr of immersion, both hydroids (Plates 13.a. and 13.b.) and bryozoa (Plate 14.a.) were present. By the third week, Celleporaria sp. (Plate 14.a.) and Membranipora sp. (Plate 14.b.) were common. Curious, stolon-like filaments (Plate 15.b.) were observed lying upon a Membranipora colony.

Protozoa were not observed upon any disc except those which had been immersed for 3 weeks. They were stalked and frequently colonial (Plates 16.a. and 16.b.).

Among the most interesting of the organisms observed were various unidentified forms. They consisted of a single colony of about 50 stalked ovoids (Plate 17.a.) indistinguishable from those discovered upon the aluminum discs, and a number of stalked, tentacled organisms, possibly ciliated protozoans, of very small size (Plates 17.b., 18.a., and 18.b.).

Algae other than diatoms were not observed.

It is worthy of note that the 3-week discs were not treated with osmium oxide but little difference was apparent between their photographs and those of the remaining discs.

VI. CONCLUSIONS AND RECOMMENDATIONS

The composition and succession of microfouling in Monterey Harbor follows very generally the pattern noted by observers in various laboratories in other parts of the world. For reasons still unknown, however, most microfoulers arrived later than expected. Glass slides immersed during this period in conjunction with independent research and examined optically were heavily colonized by bacteria within hours.

Although little microfouling was observed upon the aluminum, brass, and copper surfaces, the following inferences may be made. Either those forms sufficiently small to be obscured by the encrusting film did not attach and grow or they were encased by the collapse of the flocculent corrosion products and were hidden. Those organisms, e.g., bryozoa and hydroids, and debris too large to be covered by this corrosion crust were also absent, implying that the crust either made their attachment so fragile that they were lost, undetected, during processing or they arrive significantly later upon aluminum, brass, and copper than they do upon stainless steel.

The succession of forms upon the stainless steel followed in general that anticipated, i.e., bacteria and diatoms preceded the protozoa and metazoa. It must be concluded, however, that upon the test surfaces, at the site in question, and during the time of the experiment, the delineation of successive colonization phases was unclear, that certain anticipated steps were omitted, and other steps were reversed. Bacteria, solitary diatoms, and colonial diatoms arrived over the same time frame, during the first 2 days. During this period, however, none were present

in excessive numbers. Hydroids and bryozoa appeared within four days, settling and growing upon surfaces not previously conditioned by large numbers of bacteria and diatoms, demonstrating that the presence of an established primary film is not absolutely prerequisite to the formation of macrofouling. Protozoa appeared well after the first metazoa and, perhaps, did require the presence of large numbers of bacteria and diatoms which were lacking on discs immersed less than three weeks.

Osmium oxide did not appear to markedly improve the quality of the photographs obtained and, in view of its very hazardous nature, it is recommended that it only be used in situations requiring the finest resolution and shading. Even then, it should only be used in those places having adequate safety equipment and possessing means of disposing of its residue.

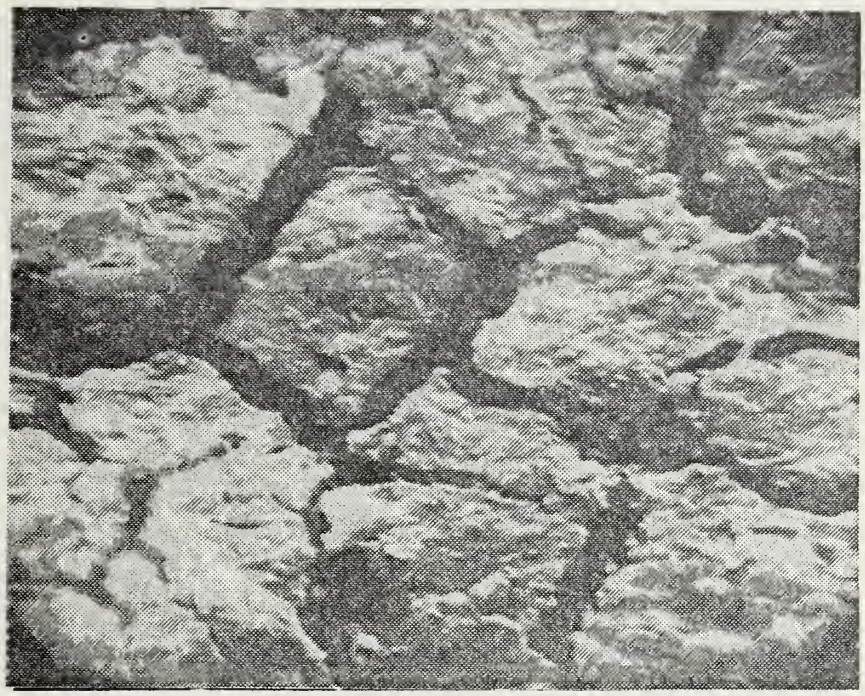

Plate 1.a. Aluminum immersed 4 hrs, 6500X. Encrustment which obscured microscopy.

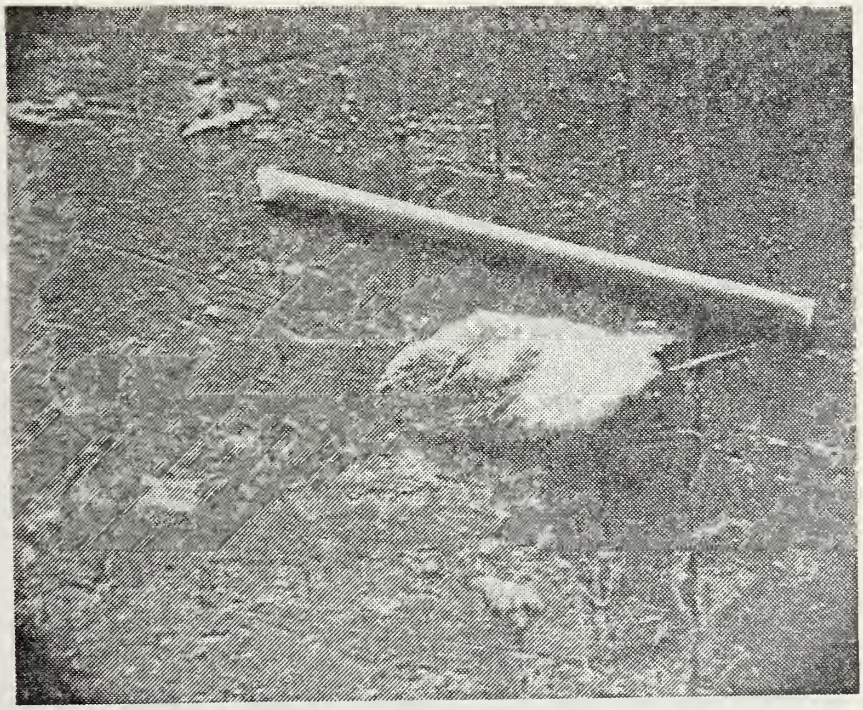

Plate 1.b. Brass immersed 4 hrs, 1260X. Debris. Note encrustment upon metal surface.

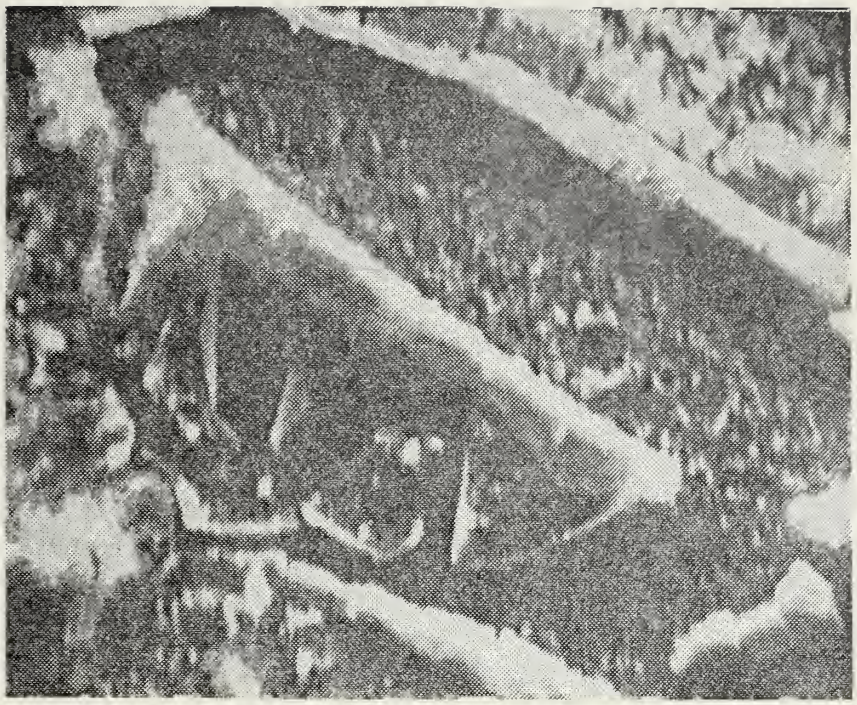

Plate 2.a. Copper immersed 1 week, 1170X. Cracked and peeling crust.

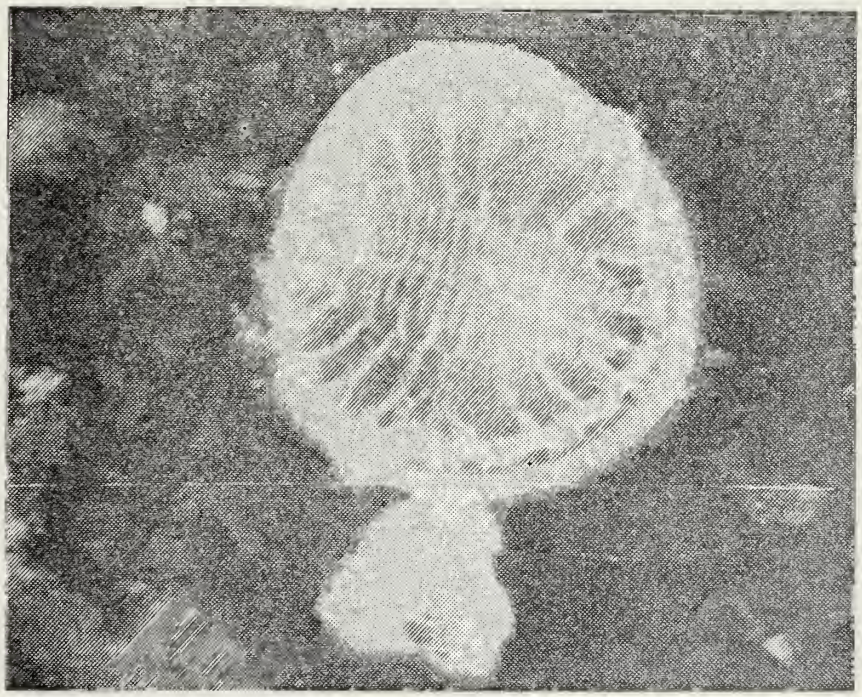

Plate 2.b. Aluminum immersed 3 weeks, 2230X. Solitary diatom.

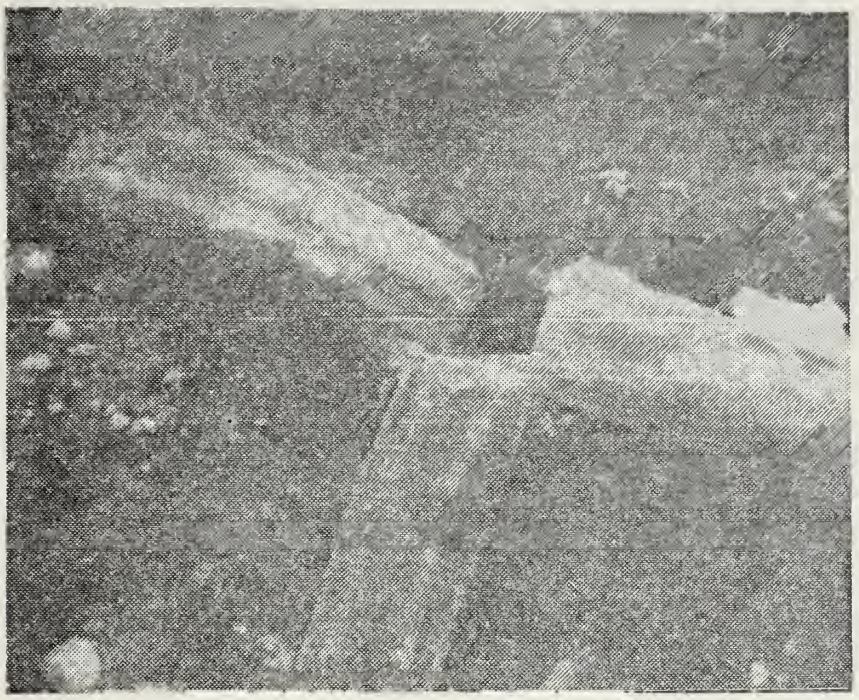

Plate 3.a. Aluminum immersed 4 days, 1200X. Chain-aggregate diatoms.

Plate 3.b. Aluminum immersed 3 weeks, 225X. Unidentified stalked, colonial organisms.

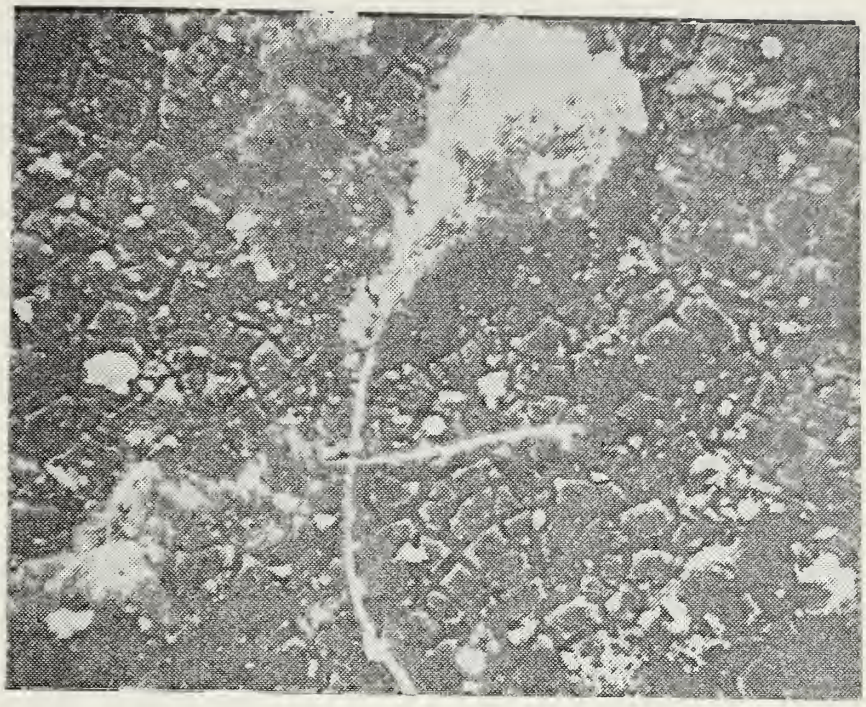

Plate 4.a. Aluminum immersed 3 weeks, 550X. Unidentified stalked, colonial organism.

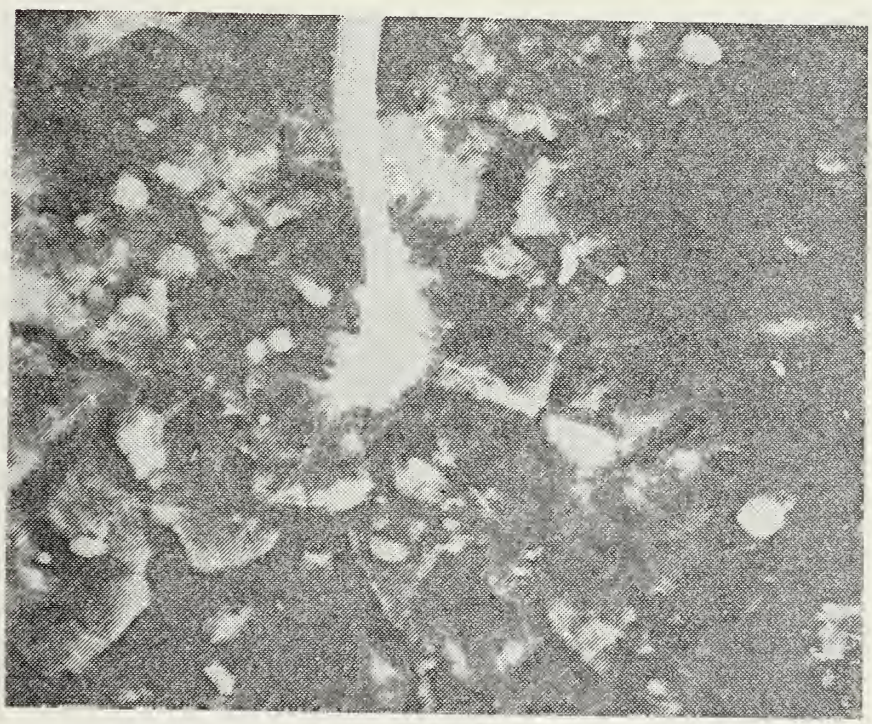

Plate 4.b. Aluminum immersed 3 weeks, 2200X. Holdfast of unidentified stalked, colonial organism.

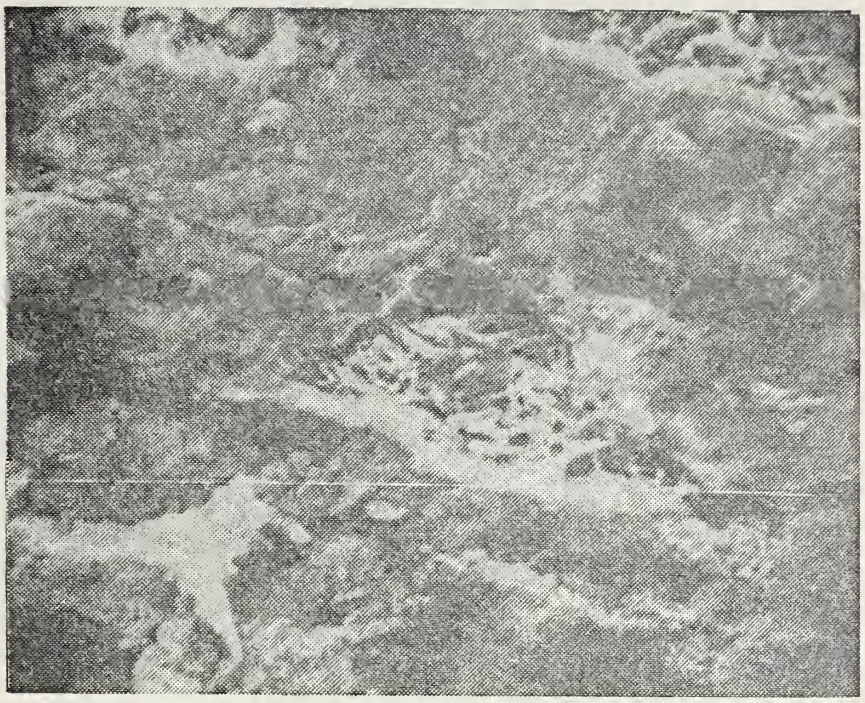

Plate 5.a. Brass immersed 3 weeks, 1130X. Layered structure of crust.

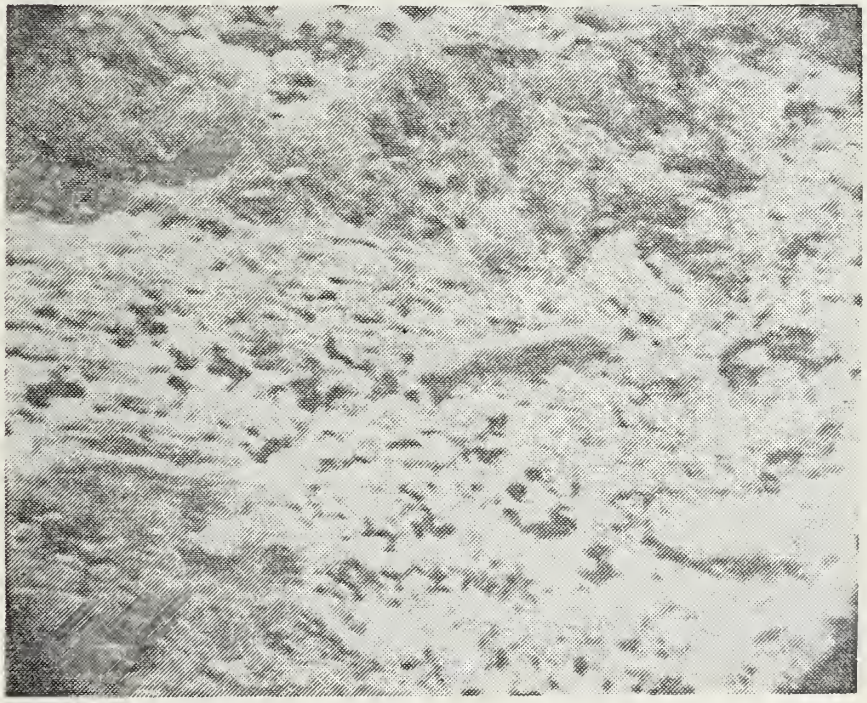

Plate 5.b. Brass immersed 48 hrs, 6200X. Bacterium.

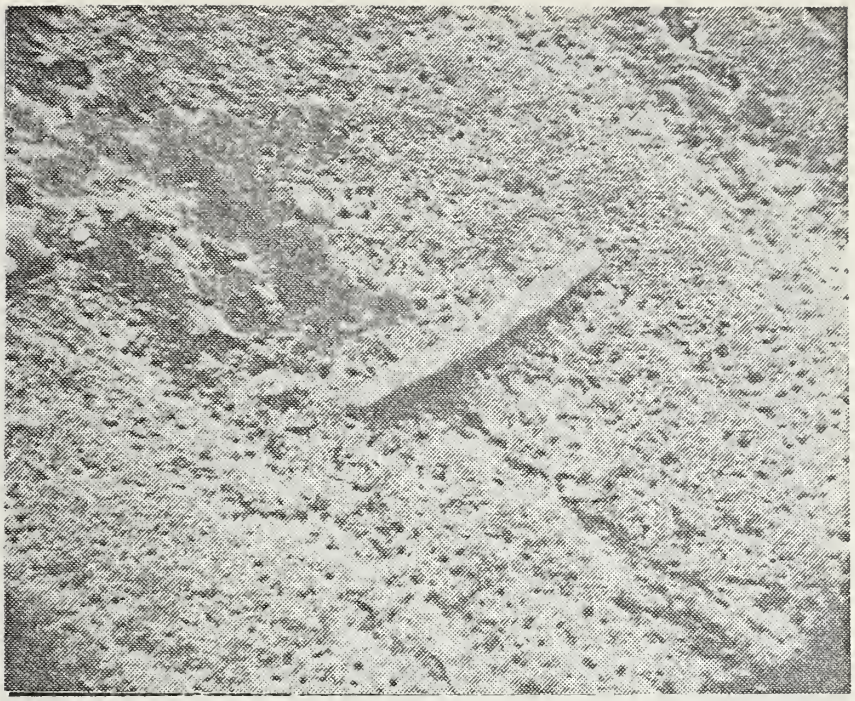

Plate 6.a. Copper immersed 4 hrs, 2400X. Unidentified object, possibly an organism.

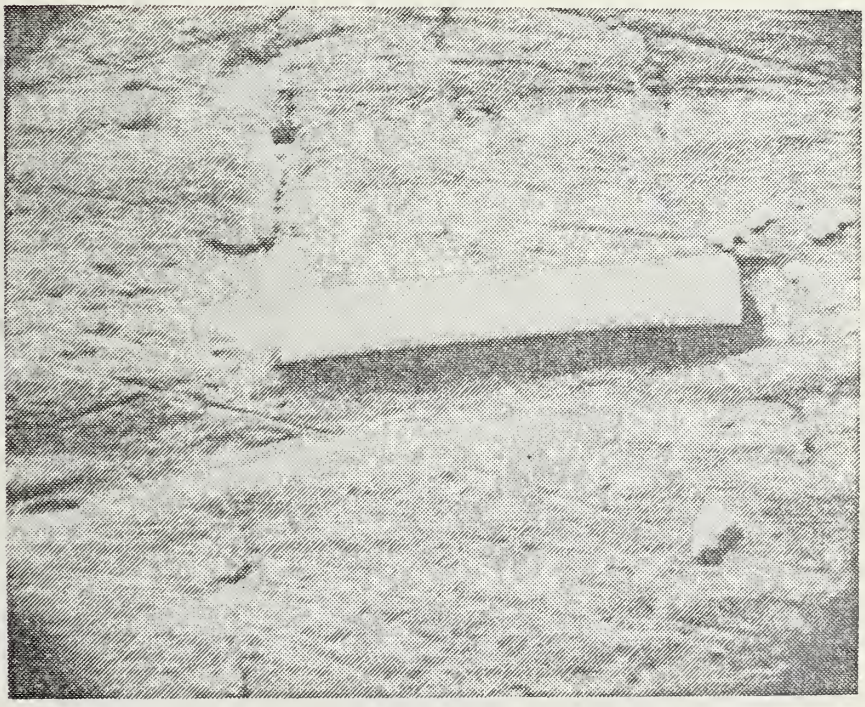

Plate 6.b. Stainless steel immersed 4 hrs, 5400X. Unidentified object, possibly a small diatom.

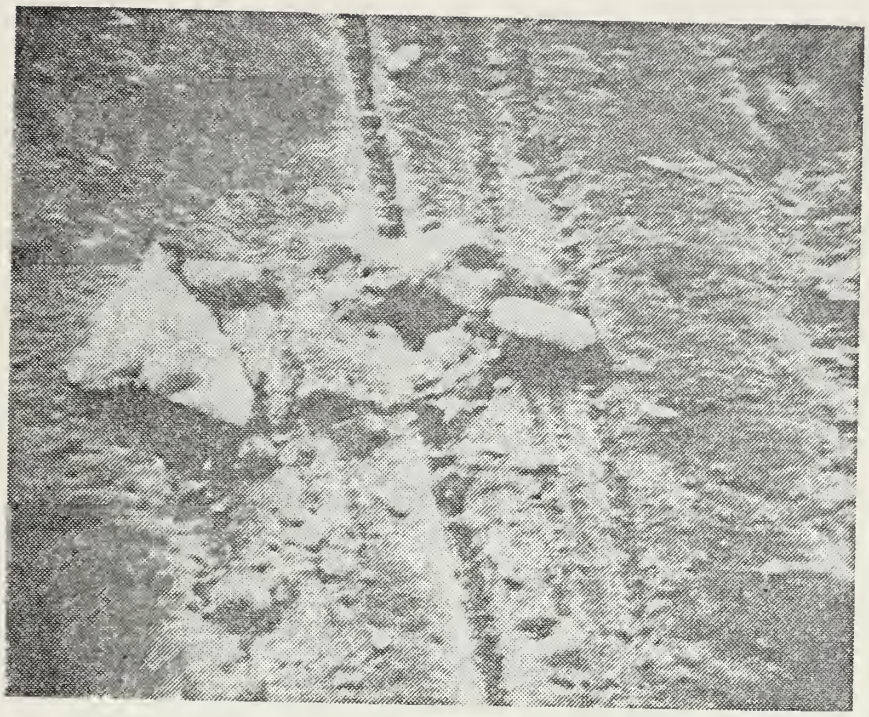

Plate 7.a. Stainless steel immersed 4 hrs, 6200X. Bacterium (right center).

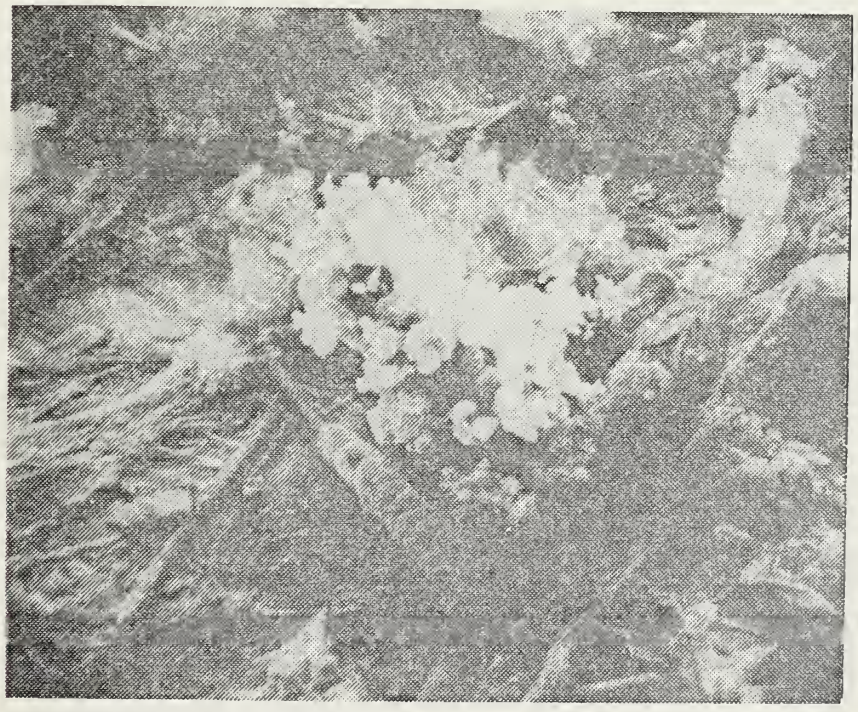

Plate 7.b. Stainless steel immersed 12 hrs, 2700X. Organic detritus. The white material in the center is believed to be a corrosion product.

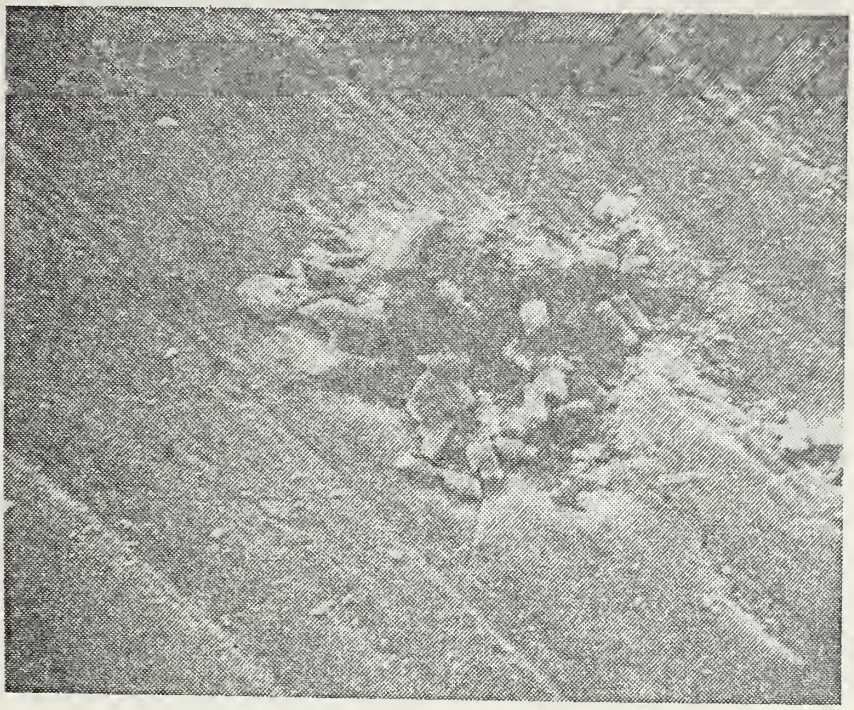

Plate 8.a. Stainless steel immersed 12 hrs, 2500X. Unidentified bodies, possibly crystals.

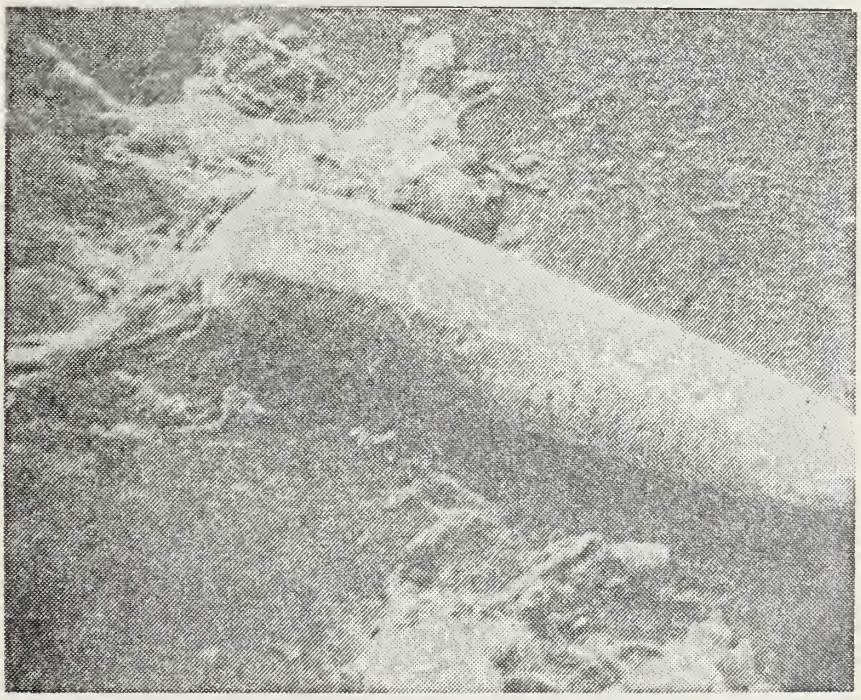

Plate 8.b. Stainless steel immersed 8 hrs, 5900X. Solitary diatom.

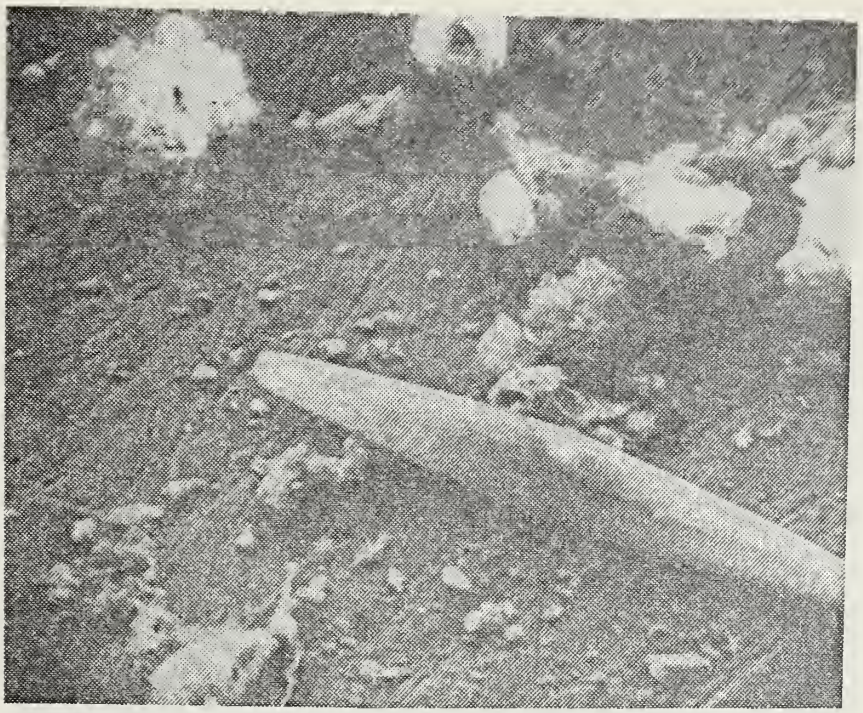

Plate 9.a. Stainless steel immersed 3 weeks, 500X. Solitary diatom.

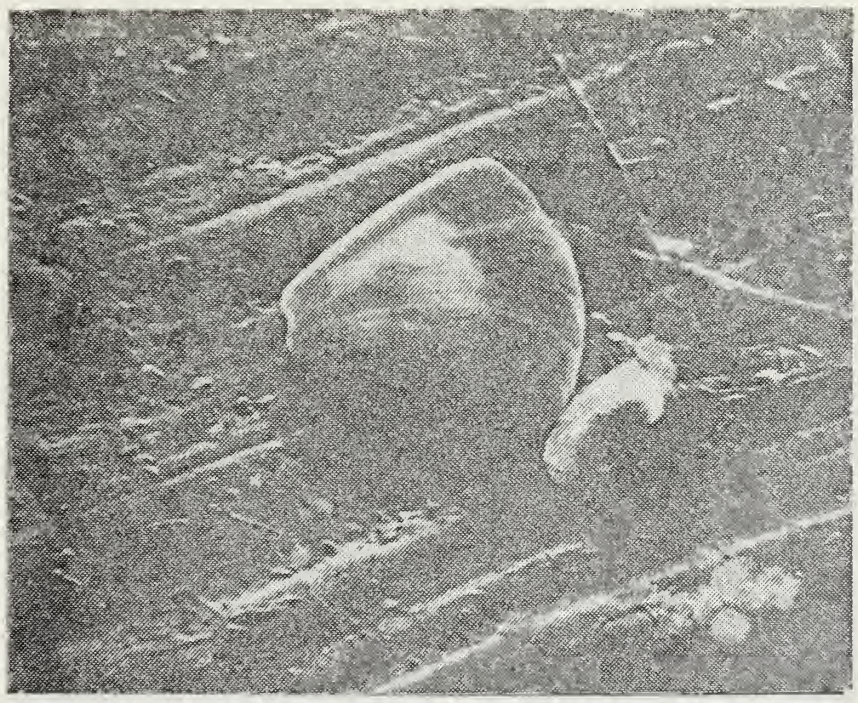

Plate 9.b. Stainless steel immersed 3 weeks, 2500X. Solitary diatom.

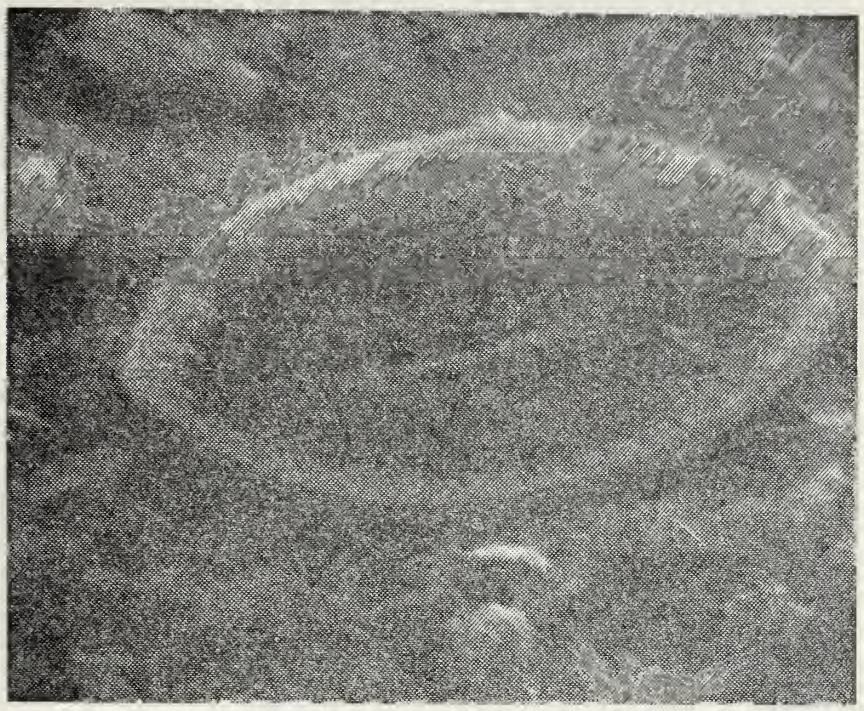

Plate 10.a. Stainless steel immersed 3 weeks, 6300X. Solitary diatom.

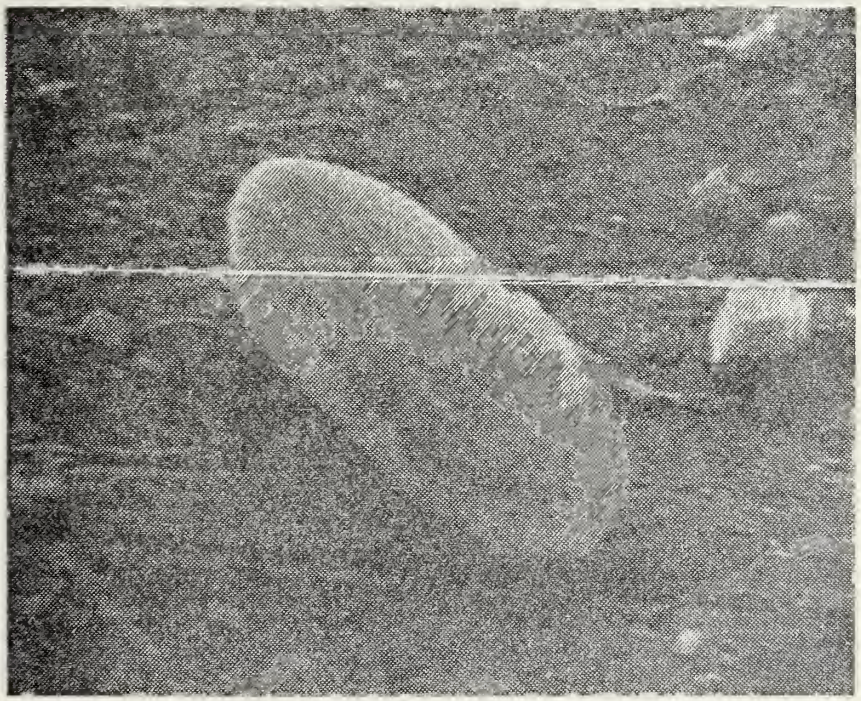

Plate 10.b. Stainless steel immersed 3 weeks, 2600X. Solitary diatom.

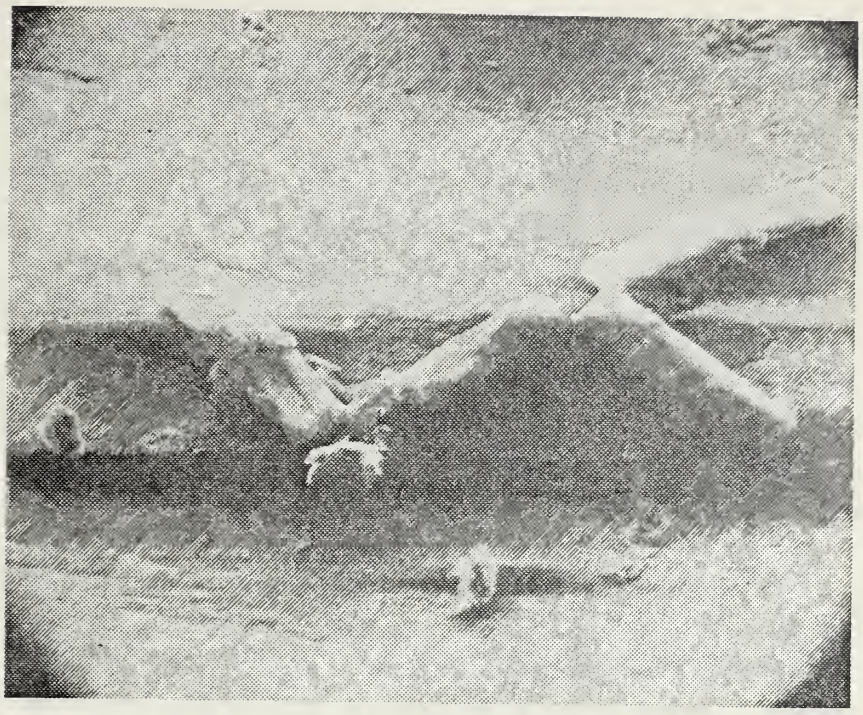

Plate 11.a. Stainless steel immersed 24 hrs, 580X. Chain-aggregate diatoms.

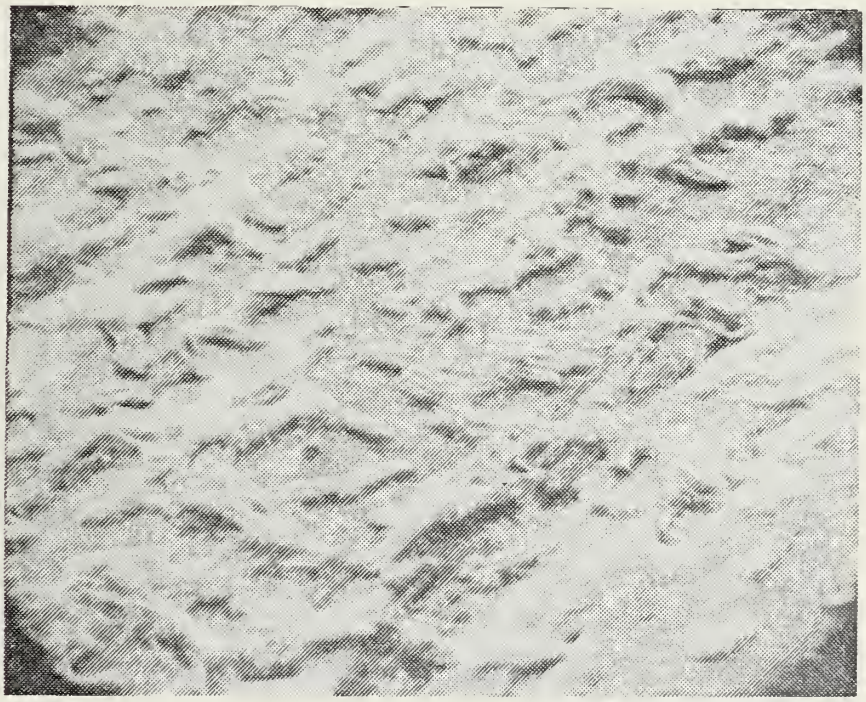

Plate 11.b. Stainless steel immersed 48 hrs, 6200X. Bacteria.

Plate 12.a. Stainless steel immersed 48 hrs, 12200X. Bacteria.

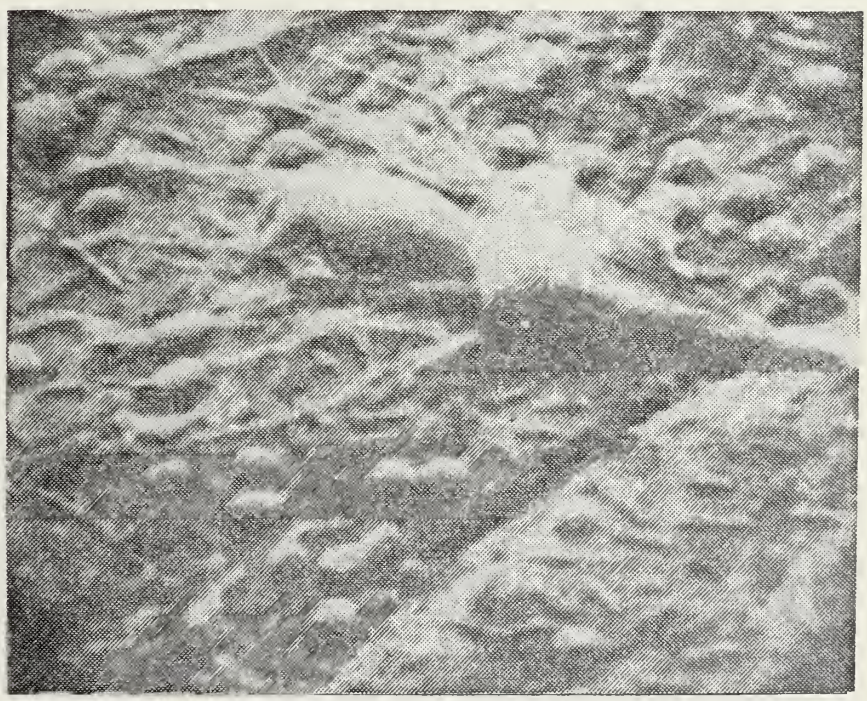

Plate 12.b. Stainless steel immersed 3 weeks, 6400X. Bacilli and cocci surrounding an unidentified, tentacled organism.

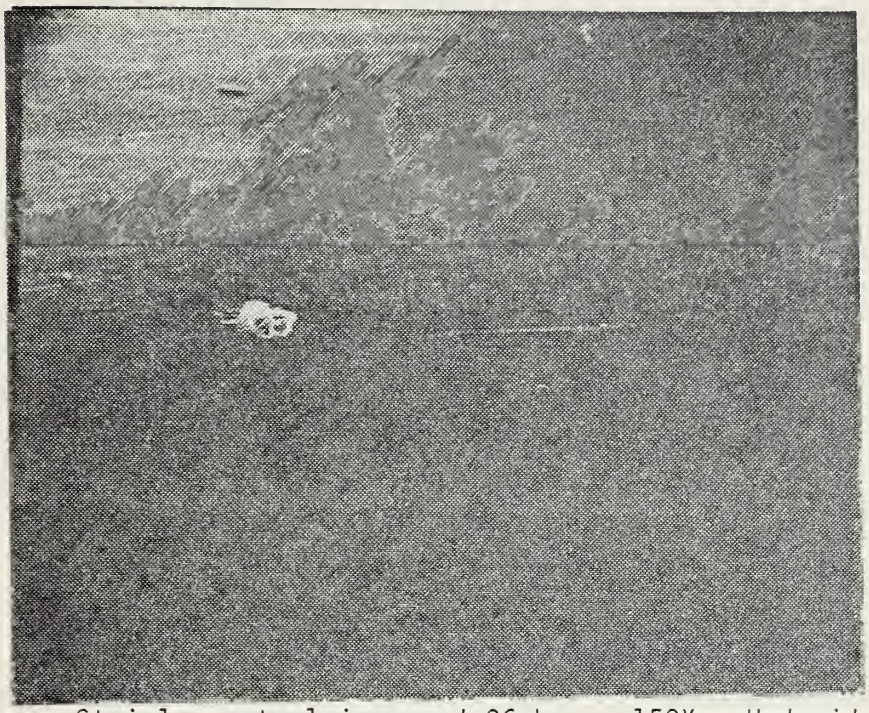

Plate 13.a. Stainless steel immersed 96 hrs, 150X. Hydroid.

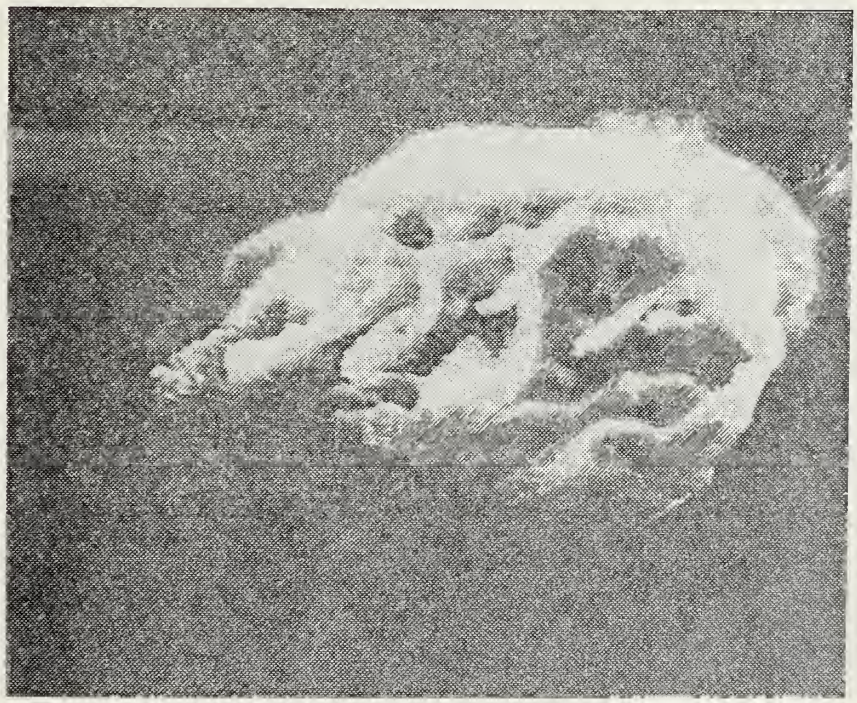

Plate 13.b. Stainless steel immersed 96 hrs, 2480X. Hydroid magnified to display tentacles.

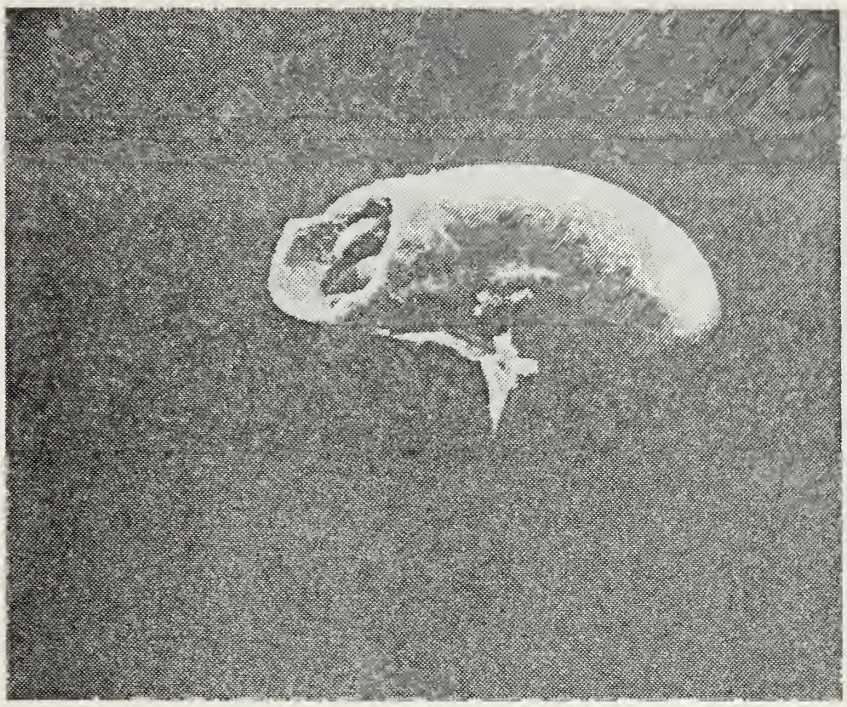

Plate 14.a. Stainless steel immersed 96 hrs, 240X. Bryozoan zooid.

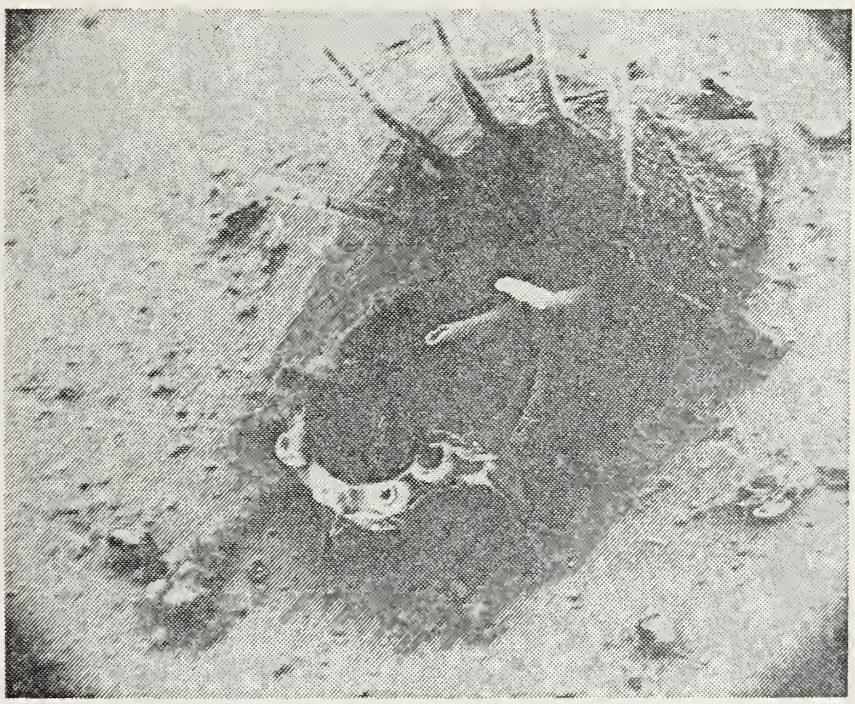

Plate 14.b. Stainless steel immersed 3 weeks, 100X. Bryozoan of the genus Celleporaria.

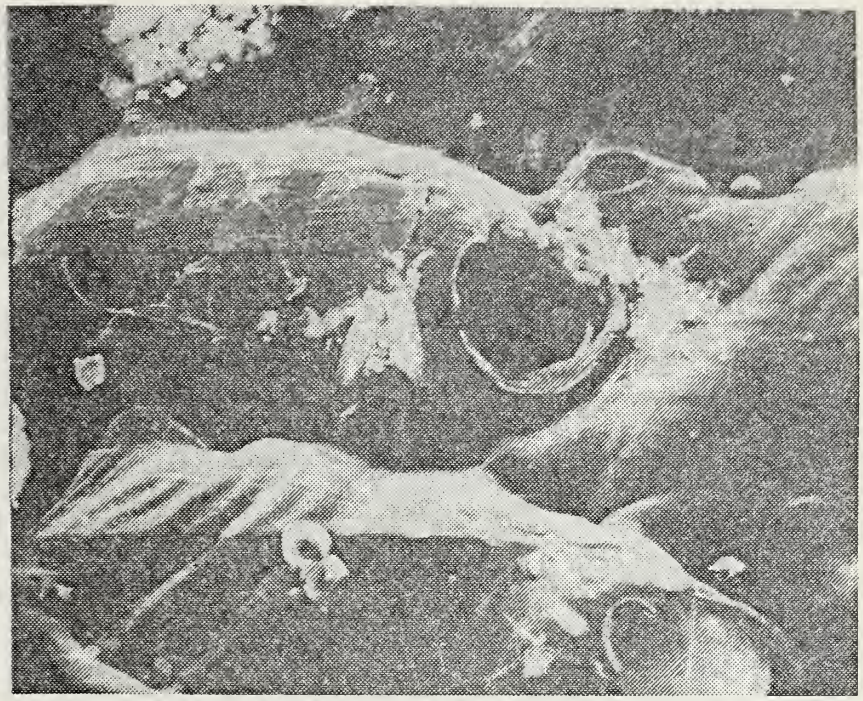

Plate 15.a. Stainless steel immersed 3 weeks, 200X. Bryozoan of the genus Membranipora.

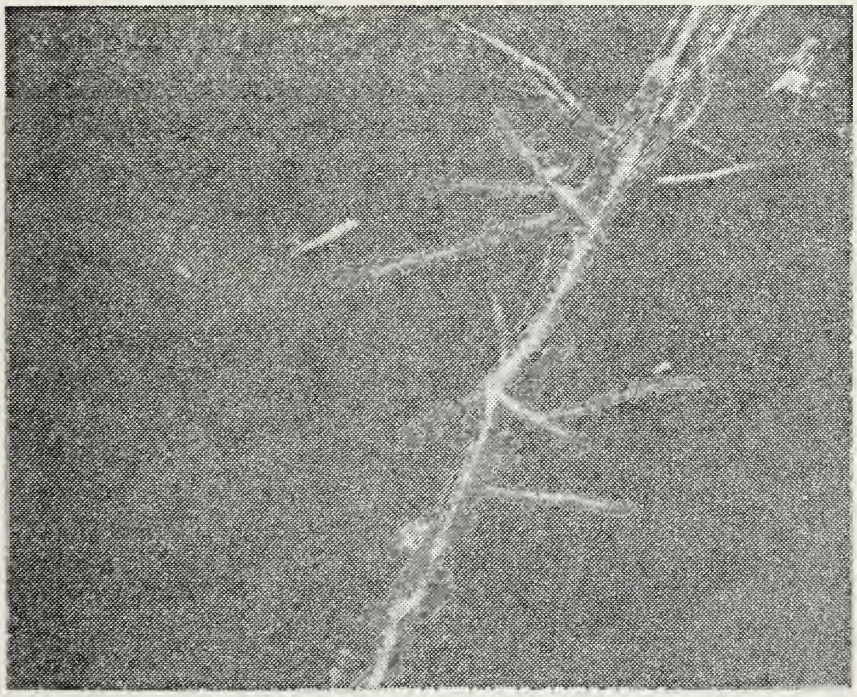

Plate 15.b. Stainless steel immersed 3 weeks, 6300X. Greater magnification of the filaments overlying the zooids in Plate 15.a.

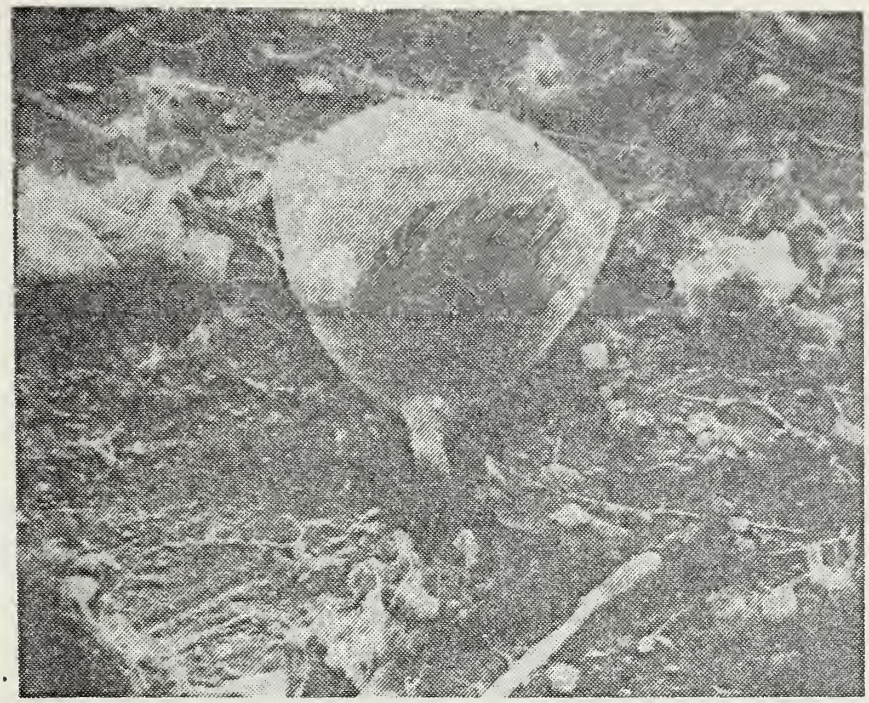

Plate 16.a. Stainless steel immersed 3 weeks, 1250X. Stalked protozoan.

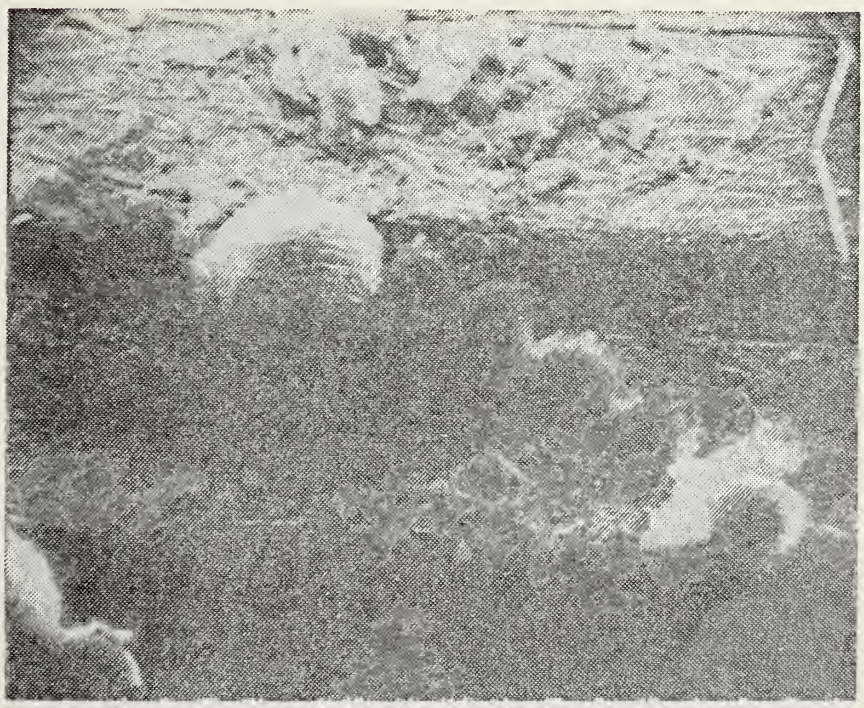

Plate 16.b. Stainless steel immersed 3 weeks, 1200X. Stalked protozoan.

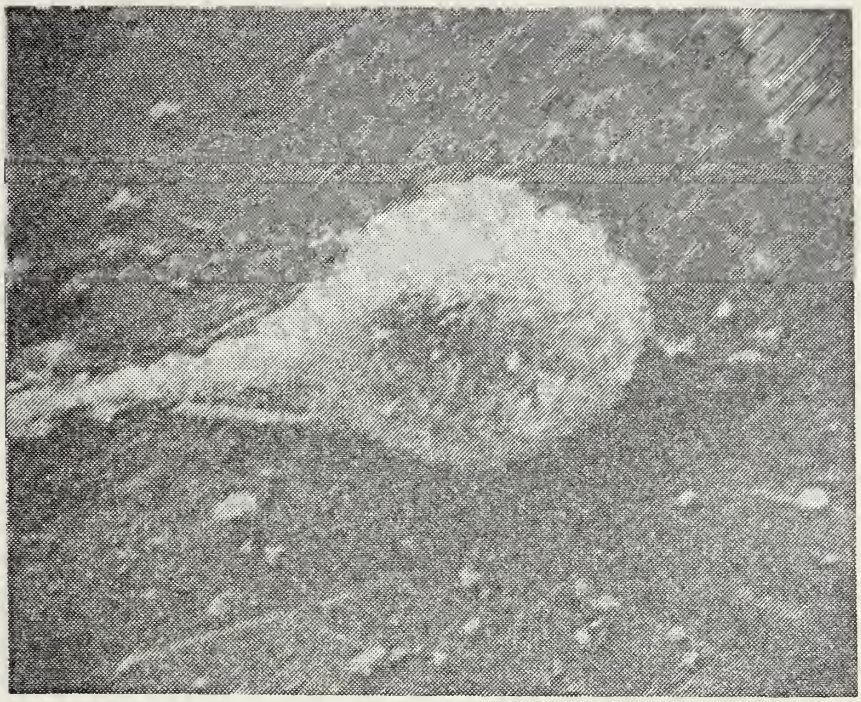

Plate 17.a. Stainless steel immersed 3 weeks, 500X. Unidentified stalked, colonial organism.

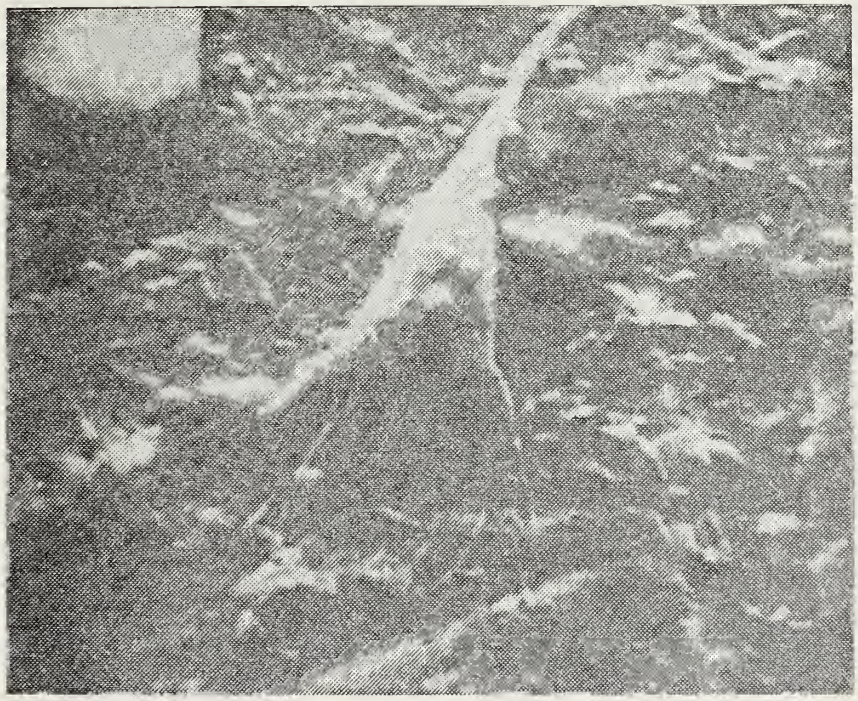

Plate 17.b. Stainless steel immersed 3 weeks, 6200X. Unidentified stalked, tentacled organism.

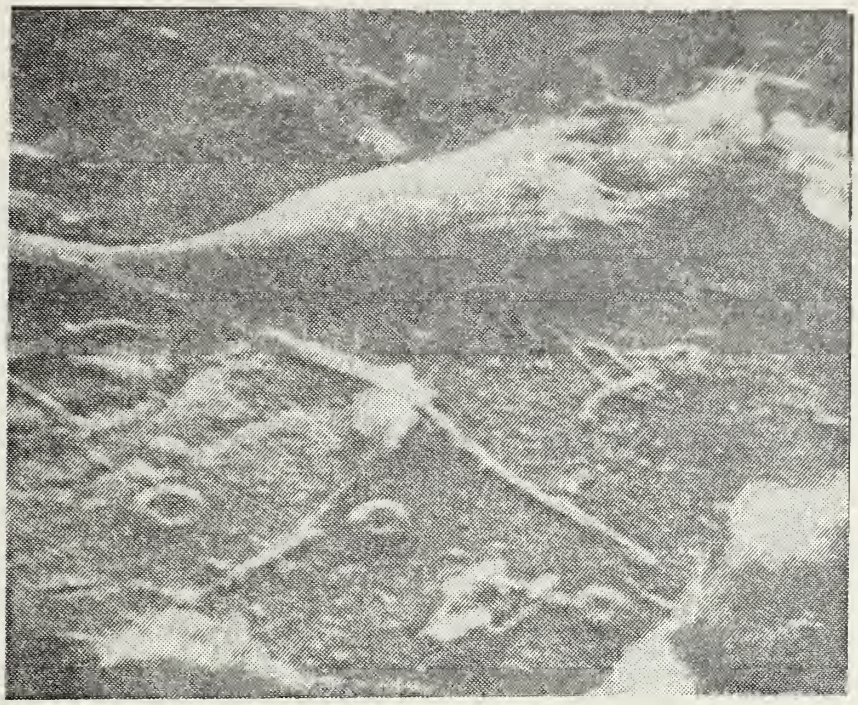

Plate 18.a. Stainless steel immersed 3 weeks, 6300X. Unidentified stalked, tentacled organism.

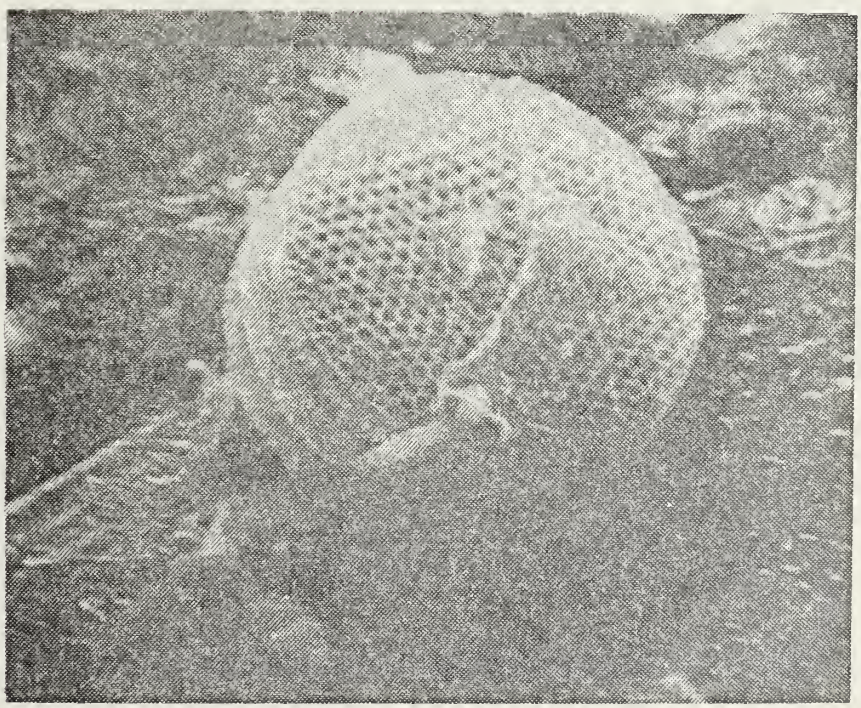

Plate 18.b. Stainless steel immersed 3 weeks, 2500X. Unidentified organisms upon a centric diatom skeleton. Note the bacteria upon the surrounding substrate.

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